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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Papisov *et al.*	Examiner:	Yong Liang Chu
Serial No:	10/521,334	Group Art Unit:	1626
Filing Date:	October 27, 2005	Confirmation No.:	1459
Title:	OXIME CONJUGATES AND METHODS FOR THEIR FORMATION AND USE		

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

APPEAL BRIEF UNDER 37 C.F.R. § 41.37

Appellant appeals to the Board of Patent Appeals and Interferences (the "Board") from the Examiner's rejection of claims 1-6, 11, 12, 14, 19, 20, 41-43, and 63-71. A Notice to this effect was filed pursuant to 37 C.F.R. § 41.31 on July 29, 2010, along with a Pre-Appeal Brief Request for Review. The Notice and Request were filed electronically at www.uspto.gov and Appellant received an Electronic Acknowledgement Receipt indicating that the Notice was received by the Patent and Trademark Office on July 29, 2010.

A Notice of Panel Decision from Pre-Appeal Brief Review was mailed on September 7, 2010, indicating that the application remains under appeal because there is at least one issue for appeal. The deadline for responding to the Notice was October 7, 2010. A Petition for a one (1) month extension of time up to and including November 7, 2010, and the extension fee of $65.00 pursuant to 37 C.F.R. § 1.17(a)(1) has been provided using the USPTO's Electronic Filing System credit card payment option. Applicant submits that since November 7, 2010 falls on a Sunday, the next succeeding day which is not a Saturday, Sunday, or Federal Holiday shall be considered timely under 37 C.F.R. § 1.7, and thus Applicant submits that the filing of this Response on Monday, November 8, 2010, is timely. The fee of $270.00 pursuant 37 C.F.R. §

41.20(b)(2) for an Appeal Brief has also been provided using the USPTO's Electronic Filing System credit card payment option.

A Table of Contents is provided for the convenience of the Board on page **3**.

Table of Contents

Real parties in Interest

As a result of assignments by the inventors, the real party in interest in this application is The General Hospital Corporation. The assignments to The General Hospital Corporation were recorded in the Patent and Trademark Office on October 20, 2005, at Reel 016667, Frame 0748. The General Hospital Corporation has licensed the subject matter of this application to Mersana Therapeutics and to the National Institutes of Health (NIH), U.S. Dept. of Health and Human Services (DHHS), U.S. Government. A confirmatory license to the National Institutes of Health was recorded in the Patent and Trademark Office on July 9, 2010, at Reel 024658, Frame 0207.

Related Appeals and Interferences

No other appeals or interferences are known to Appellant, Appellant's legal representative, or Appellant's assignee that will directly affect or be directly affected by the Board's decision in this appeal. Similarly, no such appeals or interferences are known that may have a bearing on the Board's decision in this appeal.

Status of Claims

Seventy-one (71) claims have been filed in this case. Claims 7-10, 13, 15-18, 21-41, 52-53, 57, and 60-62 have been cancelled. Claims 44-51, 54-56, and 58-59 have been withdrawn.

Claims 1-6, 11, 12, 14, 19, 20, and 41-43 were rejected in Office Actions mailed February 4, 2008, October 24, 2008 (final rejection), July 15, 2009, and April 30, 2010 (final rejection). Claims 63-71 were rejected in the Office Action mailed April 30, 2010 (final rejection).

The rejection of claims 1-6, 11, 12, 14, 19, 20, 41-43, and 63-71 is hereby appealed. A listing of pending claims 1-6, 11, 12, 14, 19, 20, 41-43, and 63-71 is provided in the **Claims Appendix** beginning on page 20.

Status of Amendments

No amendments have been filed but not entered. The pending claims (as shown in the **Claims Appendix** on page 20) reflect those submitted with the Response filed January 15, 2010, and entered by the Examiner.

Summary of Claimed Subject Matter

Independent claim 1 relates to conjugates comprising a pharmaceutically useful modifier, a carrier selected from polyketals or polyacetals, and an oxime-containing linker. Support for claim 1 is found in original claim 1 and the specification as originally filed, *inter alia*, on pages 3-4, paragraph [0010]; page 21, paragraph [0021] (for a pharmaceutically useful modifier); pages 36-37, paragraph [0082] (for a polyacetal carrier); page 37, paragraph [0083] (for a polyketal carrier); and page 39, paragraph [0091] (for molecular weight of polyal carrier).

Support for dependent claim 12 can be found in original claim 12 and the specification as originally filed, *inter alia*, on page 37, paragraph [0083].

Ground of Rejection to be Reviewed on Appeal

The ground of rejection to be reviewed on appeal is (referring to the Office Action mailed February 4, 2008):

(1) are claims 1-6, 11, 12, 14, 19, 20, 41-43, and 63-71 unpatentable under 35 U.S.C. § 103(a)?

(2) are claims 1-6, 11, 12, 14, 19, 20, and 41-43 unpatentable under nonstatutory obviousness-type double patenting over claims 29-42 of copending Application No. 10/501,565, in view of Cervigni, the '037 patent, and Hermanson?

Grouping of Claims

For the reasons discussed below in the Argument section, the claims stand or fall together for purposes of grounds of rejection numbered (1) and (2) above, as indicated below:

(1) Claims 1-6, 11, 14, 19, 20, 41-43, and 63-71 stand or fall together.

(2) Claim 12 stands or falls alone.

Argument

Introduction

Appellant must admit some frustration with the prosecution of this case. There have only been four rejections (and one provisional rejection) levied during the entire prosecution, three of which were withdrawn in the second Office Action. The fourth rejection, for obviousness, has been maintained through *four* Office Actions, but for increasingly unclear and improper reasons. Appellant has endeavored to work with the Examiner to address his concerns, including having a telephonic interview during which the Examiner indicated that proposed amendments or evidence would indeed be helpful, and subsequently providing declaratory evidence. Unfortunately, it seems impossible for Appellant to succeed in overcoming the rejection because the Examiner now (i) impermissibly uses the content of Appellant's own specification as basis for the obviousness rejection, and (ii) dismisses a demonstrated state of the art at the time the present application was filed in his arguments maintaining the obviousness rejection.

Appellant below summarizes the prosecution history of this case with regard to the obviousness rejection, in order to illustrate the evolving nature of the maintained rejection, as well as the thoroughness of Appellant's responses to each articulation of the rejection. As will be clear, Appellant has more than satisfied the legal requirements for nonobviousness, the present claims are patentable to Appellant, and the rejections should be reversed.

Claims 1-6, 11, 12, 14, 19, 20, 41-43, and 63-71 are not obvious over the cited references

The presently pending claims relate to conjugates comprising a pharmaceutically useful modifier, a carrier selected from polyketals or polyacetals, and an oxime-containing linker.

The specification describes the synthesis of exemplary bifunctional reagents that may be used to form the claimed conjugates, and also describes the conjugation of proteins to carriers, wherein the resulting conjugates have an oxime-containing linker. Additional data are provided on blood clearance, hydrolytic stability, enzymatic stability, biokinetics, and biodistribution of such conjugates. The present Appellant discovered, among other things, that despite the state of the art prior to Appellant's disclosure that indicated otherwise, it is possible to use oxime

chemistry to make conjugates comprising a polyketal or polyacetal carrier. The specification enables this finding, as recited in the present claims.

The initially examined claim 1 referred to a conjugate comprising a carrier substituted with one or more occurrences of a biologically active modifier, wherein the modifier is linked to the carrier via an oxime-containing linker.

In an Office Action ("the first Office Action") mailed on February 4, 2008, the Examiner issued rejections under (i) 35 U.S.C. § 112, first paragraph, for failure to comply with the written description requirement, (ii) 35 U.S.C. § 112, second paragraph, for indefiniteness, (iii) 35 U.S.C. § 102(b), for anticipation by Cervigni *et al.*, *Angew. Chem. Int. Ed. Engl.*, (1996), 35(11), pp. 1230-1232 ("Cervigni *et al.*"), and (iv) 35 U.S.C. § 103(a) for alleged obviousness over Cervigni *et al.* in view of U.S. Patent No. 5,958,398 by Papisov ("the '398 patent"), U.S. Patent No. 5,612,037 by Huebner ("the '037 patent"), and G. Hermanson, Preparation of Liposome Conjugates and Derivatives, Bioconjugate Techniques, pp. 552-589, ("Hermanson"). The first three rejections were easily overcome by Appellant's arguments and amendments and will not be discussed further here. A provisional double patenting rejection over copending U.S. Pat. Application No. 10/501,565 was also levied in the first Office Action and is maintained as far as Appellant can tell.

Regarding the obviousness rejection, the Examiner stated that "Cervigni *et al.* teach an oxime conjugate of a carrier and modifier, but do not teach all the specific carrier such as polyketal as in claims 12 and 14; maleiimide- or N-hydroxysuccinimide ester containing crosslinker as in claims 3 and 4; or a liposome based carrier." The Examiner indicated that such differences would have been obvious to one skilled in the art over the combined teachings of the cited references.

Cervigni *et al.* describes conjugates of a saccharide and a peptide, comprising an oxime. A saccharide, of course, is chemically entirely different from a polyacetal or polyketal. Notably, the oxime bonds in the Cervigni *et al.* conjugates are not even between the saccharide and peptide moieties. Rather, oxime bonds are formed between the peptide and a decanal moiety, while the bond between the peptide and saccharide moieties present in Cervigni *et al.* is a hydroxylamine ether bond, which is entirely different from an oxime.

The Examiner constructed an obviousness rejection of the claimed polyacetal/ketal-oxime-modifier conjugates over Cervigni *et al.*'s conjugates merely by pointing out that the '398 patent describes preparation and conjugation of polyacetals and polyketals. The Examiner did not even try to explain why one of ordinary skill in the art might be motivated (1) to use something other than a saccharide in Cervingi *et al.*'s system; (2) to select a polyacetal or polyketal; and (3) to make an oxime bond in a *different location* than Cervigni *et al.,* did. Furthermore, the Examiner assumed success without ever addressing the very different chemistries involved.

In a Response filed August 4, 2008 ("the first Response"), Appellant pointed out each of these failures of motivation. Specifically, Appellants noted that Cervigni *et al.* does not offer *any* suggestion or motivation that its described conjugates should be modified; nor does the '398 patent provide *any* teaching or suggestion to motivate one of skill to attempt oxime linkages. Moreover, Appellant further explained that those of ordinary skill in the art, reading the cited references, would be strongly *motivated away* from attempting to modify the Cervigni *et al.* conjugates as proposed by the Examiner, and certainly would not have had *any* reasonable expectation of success of achieving the claimed conjugates. It is well established that it is improper to combine references where the references teach way from their combination. *In re Grasselli*, 713 F.2d 731, 743 (Fed. Cir. 1983).

Cervigni *et al* prepare their conjugates using acidic conditions and nucleophilic amino-oxy reagents (*e.g.*, O-hydroxylamines) to form oximes. In the first Response, Appellant amended the claims to recite a carrier selected from polyacetals or polyketals. It was known at the time that the present application was filed that the integrity of polyacetals (such as those described in the '398 patent) can be compromised under such acidic conditions, and particularly in the presence of amino-oxy reagents. Appellant explained these facts in the first Response and submitted that the amended claims were non-obvious and should be allowed[1].

[1] Appellant also made certain points in the first Response explaining that the proposed combinations actually taught away from certain dependent claims, but those claims have been cancelled and will not be addressed further here. Also, the first Response addressed Hermanson, which was apparently applied only against claim 22. Claim 22 is also no longer pending and those points will not be discussed further.

In a second, and Final Office Action ("the second Office Action") mailed October 24, 2008, the Examiner maintained the obviousness rejection, asserting that "a conjugate of polyacet[al] or polyketals with a modifier through an oxime-containing li[n]ker are taught, suggested and motivated from the combined references" and "in terms of the argument of a reasonable expectation of success [...] a reasonable stability of polyacet[al] or polyketals from nucleophilic attack is predictable to one ordinary skilled in the art by choosing appropriate reaction conditions without undue experimentation, because they are known to one skilled in the art, and taught by many references. In addition, polyacetyls [sic] or polyketals **are not such labile** under neutral pH condition" (emphasis original).

In a Response ("the second Response") filed April 24, 2009, Appellant noted that the *stability of polyacetals at neutral pH is irrelevant* to a proper obviousness rejection. The cited references did not describe oxime conjugate formation at neutral pH. The *only* reaction conditions provided in the cited references for formation of oxime bonds were the *very acidic* conditions of Cervigni *et al.* As part of the second Response, Appellant submitted *data,* published *prior to Appellant's priority date* that showed pH-dependent stability of an exemplary polyacetal, PHF or poly(hydroxymethylethylene hydroxymethylformal). Specifically, under nearly identical conditions taught by Cervigni *et al.* (pH 3 for 120 hours), PHF was shown to undergo nearly complete hydrolysis of the polymer main chain (see **Exhibit C**, Figure 4, PHF after 4 days incubation at pH 3).

Appellant further demonstrated that a person of ordinary skill in the art, reading Cervigni *et al.* and the '398 patent, would (1) not be motivated to perform the conjugation described by Cervigni *et al.* on the polyacetals of the '398 patent (due, for example, to known instability problems of polyacetals described in the '398 patent under acidic conditions), and (2) would not have a reasonable expectation of success of preparing the claimed conjugates even if so motivated. Indeed, Appellant pointed out that those of ordinary skill in the art, reading Cervigni *et al.* and the '398 patent in the context of the art at the time, would have appreciated that use of the Cervigni *et al.* conditions with the '398 patent compounds would not generate the claimed conjugates, but rather would result in nearly complete hydrolysis of the PHF main chain.

A third Office Action ("the third Office Action") mailed July 15, 2009, the Examiner maintained the obviousness rejection, asserting that Cervigni *et al.* teaches alternative, more mild conditions, and cited yet another reference, "Rose" (Rose, *J. Am. Chem. Soc.* (1994), 116, pp. 30-33).

In the Response ("the third Response") filed January 15, 2010, Appellant explained that the conditions in fact described by Cervigni *et al.* were not actually "mild" with respect to PHF. Moreover, Appellant provided *evidence*, in the form of a Declaration under 37 CFR § 1.132,[2] executed by an inventor (Dr. M. Papisov) of the present invention further explaining the state of the art at the time the present application was filed, and explaining how one of ordinary skill of the art ***who did not have the benefit of the present specification*** would have understood the teachings of Cervigni *et al.* and the '398 patent. As set forth in detail in the Declaration, at the time the instant application was filed, the state of the art—at best—indicated a significant level of unpredictability regarding the reactivity and stability of polyacetals. Appellant explained why, in light of the state of the art, it would not have been obvious to one of ordinary skill in the art to combine the Cervigni *et al.* (or Rose) oxime-forming conditions with the polyacetals of the '398 patent.

The Declaration explains several reasons why there would be no motivation to combine the Cervigni *et al.* methods with the polyacetals of the '398 patent and/or why there would not be a reasonable expectation of success even if combined. First, the literature on polyacetals indicated hydrolysis at acidic pH (see paragraph 10 of Declaration) that may occur through at least two mechanisms (see paragraphs 10-11 of Declaration). Second, the Cervigni *et al.* and

[2] In a telephonic interview on December 7, 2009, Appellant and Examiner discussed the state of the art at the time of filing, the cited art, and possible amendments to the claims. In particular, Inventor M. Papisov, Ph.D., explained to the Examiner how one of ordinary skill could not have predicted at the time the present application was filed that polyacetals or polyketals would be able to form the claimed conjugates due to their unpredictable reactivity and stability. Dr. Papisov further explained how the polymeric carrier is susceptible to hydrolytic degradation under acidic conditions, and an amendment to the claims was discussed in this context. The Examiner seemed to understand the scientific issues at hand and requested that Appellant provide the contents of the discussion in a Declaration under 37 CFR § 1.132.

Rose methods are described for monomeric substrates, but polymeric substrates such as polyacetals and polyketals can exhibit very different reactivity that cannot be translated from monomeric studies (see paragraphs 5-7 and 16 of the Declaration). Third, the possibility of hydrolysis of a polymeric carrier in biomedical applications is highly undesirable, and a skilled artisan concerned with the avoidance of byproducts, purity and molecular weight of a polymer conjugate would not consider subjecting a polyacetal of the '398 patent to the reaction conditions taught by Cervigni *et al.* and Rose (see paragraphs 17-18 of Declaration). Fourth, the reagents used to form an oxime, a hydroxylamine could destabilize the polymer main chain (see paragraph 14 of Declaration).

It is at this point that the Examiner appears to have decided that the patentability of the present claims turns on the question of whether, sitting here today, in full possession of the knowledge provided by the present specification, one of skill might determine that the claimed conjugates *could* be made using the oxime-forming chemistry taught by Cervigni *et al.* As an initial matter, Appellant points out that the proper obviousness analysis is not what *we now know*, in light of an Applicant's own patent application specification, to be possible, but rather whether one of ordinary skill in the *art at the time of filing* would be motivated make a particular combination and have a reasonable expectation of success in so doing. The Examiner has repeatedly used Appellant's own disclosure to allege that Appellant's claimed invention is obvious, while ignoring evidence that establishes the state of the *art at the time of filing*.

In the current Office Action mailed April 30, 2010, the Examiner refers to Appellant's Declaration under 37 CFR § 1.132 and states:

> "The key argument Applicants made here is that an oxime-containing polyketal and polyacet[al] ***is not*** stable under acidic conditions (i.e. at pH ~4) due to the stability of polyacetals or poly[ketals]. However, at lines 1-4, paragraph [0279], page 92 of the instant specification, it states that the coupling reaction between PHF diol and the couple reagent VII was carried out at pH = 3.0 by addition of 1M $NaHSO_4$ and agitated for 2 hours on the ice. This disclosure clear[ly] contradicts with the statement Applicants made in the instant 132 Declarations, namely an oxime-containing polyketals and polyacet[al] conjugate ***is not*** stable under acidic conditions" (emphasis added).

Thus, the Examiner maintains the rejection by dismissing the Declaration on the ground that *the present specification* provides information, not found in the prior art, that shows the prior art understanding/expectation in fact was incorrect and it is possible to produce the claimed invention. The Examiner thus *relies on the present invention* to reject the present claims! This reliance is impermissible. The teachings, suggestions, and expectation of success must come from the prior art, not Appellant's disclosure. *See In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991). Appellant has *demonstrated*, through submission both of published literature and of sworn declaration testimony, that those of ordinary skill in the art, reading the cited references in the absence of the present disclosure, would *not have been motivated to try* to use the Cervigni *et al.* methods to generate conjugates of the '398 compounds and, moreover, even if for some reason they were so motivated to try, would not have had a reasonable expectation of success.

The alleged contradiction between Appellant's Declaration under 37 CFR § 1.132 and the present specification is incorrect. The Declaration speaks to the state of the art prior to the filing of the present application regarding the stability of polyacetals under acidic conditions. Contrary to the Examiner's statement, the Declaration does not speak of the stability of the *claimed conjugates* under acidic conditions; rather, it indicates what would have been known to the skilled artisan at the time the present application was filed. In fact, Appellant submits that regarding the known stability of polyacetals, any difference between the state of the art at the time the present application was filed and Appellant's disclosure represents evidence of unexpected results, not contradictory statements as asserted by the Examiner.

The *present disclosure* provides the teaching, unexpected in light of the state of the art, that the claimed conjugates ***can*** be made under acidic conditions. The Examiner may not rely on the *present specification* for motivation to combine cited references to render obvious the present claims, and in doing so the Examiner has impermissibly used the content of the specification as basis for the obviousness rejection.

Furthermore, the Examiner's apparent dismissal of Appellant's Declaration on the grounds that it does not contain comparative data is unreasonable if not illogical, particularly in the instant case where the utility of comparative data isn't even clear. The purpose of the

provided Declaration was to describe the state of the art prior to Appellant's disclosure, not compare the prior art conjugates with Appellant's conjugates.

For all of these reasons, Appellant respectfully submits that the rejection under 35 U.S.C. § 103(a) is improper and therefore the Examiner has not established a *prima facie* case of obviousness with respect to claims 1-6, 11, 12, 14, 19, 20, 41-43, and 63-71.

Claim 12

The '398 patent describes polyacetals, but not polyketals, and none of the other references relied upon in the rejection under 35 U.S.C. § 103(a) describes polyketals as recited in claim 12. Therefore, claim 12 is separately patentable.

Double Patenting

In the first and second Office Actions, a provisional obviousness-type double patenting rejection was levied against claims 1-12, 14, 19-22, and 41-43 for being unpatentable over claims 29-42 of copending Application No. 10/501,565 (the '565 application) in view of Cervigni *et al.*, the '037 patent, and Hermanson. In response, Appellant requested that this provisional rejection be held in abeyance to be addressed when a relevant claim of the '565 application issues. While not mentioned in the two most recent Office Actions, it appears this rejection is still of record and Appellant believes the rejection is improper for at least the following reason:

i) The Examiner did not establish a prima facie case of obviousness regarding the combination of claims 29-42 of the '565 application with the other references in the first Office Action.

In the rejection, the Examiner states that "the '565 application claims a biodegradable biocompatible polyketal polymer [...] with a nitrogen-containing moiety" and that using a "polyketal as a carrier of the conjugate in claims 12 and 14 has been claimed in the '565 application as a biodegradable polyacet[al] polymer [...] and suggested to crosslink with a drug

to form a conjugate." The Examiner is incorrect. The word "crosslink" does not appear in claims 29-42 of the '565 application, although it does appear in claim 27 of the '565 application. Even so, the Examiner has not established why (or how), in view of Cervigni *et al.*, one of ordinary skill in the art would be motivated to select "pharmaceutically useful group" (*i.e.*, drug) and "nitrogen-containing moiety" from two separate claims in the '565 application, combine them into a single moiety appended and/or crosslinked to the polyketals of the '565 application, and arrive at the claimed conjugates having an oxime-containing crosslinker.

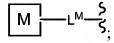
Appellant respectfully submits that the double patenting rejection is improper and therefore the Examiner has not established a *prima facie* case of obviousness with respect to claims 1-6, 11, 12, 14, 19, 20, and 41-43. Appellant reserves the right to obviate this rejection by filing a terminal disclaimer.

Claims appendix

Pending claims

(As submitted in Response filed January 15, 2010)

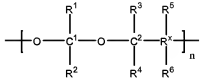
1. **(Previously Presented)** A conjugate comprising a carrier substituted with one or more occurrences of a moiety having the structure:



wherein each occurrence of M is independently a pharmaceutically useful modifier;

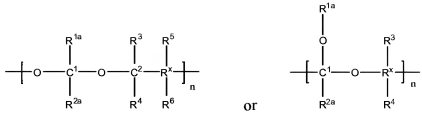
the carrier comprises a biodegradable biocompatible polymer selected from polyacetals or polyketals and the molecular weight of the carrier is between about 0.5 and about 1500 kDa;

wherein at least a subset of the polyacetal repeat structural units have the following chemical structure:



wherein for each occurrence of the n bracketed structure, one of R^1 and R^2 is hydrogen, and the other is a biocompatible group and includes a carbon atom covalently attached to C^1; R^x includes a carbon atom covalently attached to C^2; n is an integer; each occurrence of R^3, R^4, R^5 and R^6 is a biocompatible group and is independently hydrogen or an organic moiety; and for each occurrence of the bracketed structure n, at least one of R^1, R^2, R^3, R^4, R^5 and R^6 comprises a carbonyl group suitable for oxime formation;

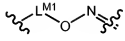
wherein at least a subset of the polyketal repeat structural units have the following chemical structure:



wherein each occurrence of R^{1a} and R^{2a} is a biocompatible group and includes a carbon atom covalently attached to C^1, and at least one of R^{1a}, R^{2a}, R^3, R^4, R^5 and R^6 comprises a carbonyl group suitable for oxime formation and

each occurrence of L^M is independently an oxime-containing linker.

2. **(Original)** The conjugate of claim 1, wherein each occurrence of L^M is independently a moiety having the structure:



wherein each occurrence of L^{M1} is independently a substituted or unsubstituted, cyclic or acyclic, linear or branched $C_{0\text{-}12}$alkylidene or $C_{0\text{-}12}$alkenylidene moiety wherein up to two non-adjacent methylene units are independently optionally replaced by CO, CO_2, COCO, $CONR^{Z1}$, $OCONR^{Z1}$, $NR^{Z1}NR^{Z2}$, $NR^{Z1}NR^{Z2}CO$, $NR^{Z1}CO$, $NR^{Z1}CO_2$, $NR^{Z1}CONR^{Z2}$, SO, SO_2, $NR^{Z1}SO_2$, SO_2NR^{Z1}, $NR^{Z1}SO_2NR^{Z2}$, O, S, or NR^{Z1}; wherein each occurrence of R^{Z1} and R^{Z2} is independently hydrogen, alkyl, heteroalkyl, aryl, heteroaryl or acyl.

3. **(Original)** The conjugate of claim 2, wherein one or more occurrences of L^{M1} independently comprises a maleimide- or N-hydroxysuccinimide ester-containing crosslinker.

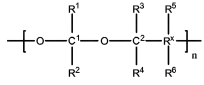
4. **(Original)** The conjugate of claim 3, wherein one or more occurrences of L^{M1} independently comprises a 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, m-maleimidobenzoyl or a 4-(p-maleimidophenyl)butyrate crosslinker.

5. **(Original)** The conjugate of claim 1, wherein one or more occurrences of M comprises, or is attached to the carrier through, a biodegradable bond.

6. **(Original)** The conjugate of claim 4, wherein the biodegradable bond is selected from the group consisting of acetal, ketal, amide, ester, thioester, enamine, imine, imide, dithio, and phosphoester bond.

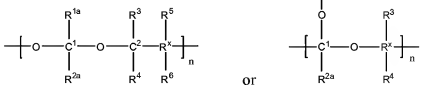
7-10. **(Cancelled)**.

11. **(Previously Presented)** The conjugate of claim 1, wherein the carrier is a biodegradable biocompatible polyacetal wherein at least a subset of the polyacetal repeat structural units have the following chemical structure:



wherein for each occurrence of the n bracketed structure, one of R^1 and R^2 is hydrogen, and the other is a biocompatible group and includes a carbon atom covalently attached to C^1; R^x includes a carbon atom covalently attached to C^2; n is an integer; each occurrence of R^3, R^4, R^5 and R^6 is a biocompatible group and is independently hydrogen or an organic moiety; and for each occurrence of the bracketed structure n, at least one of R^1, R^2, R^3, R^4, R^5 and R^6 comprises a carbonyl group suitable for oxime formation.

12. **(Previously Presented)** The conjugate of claim 1, wherein the carrier is a biodegradable biocompatible polyketal wherein at least a subset of the polyketal repeat structural units have the following chemical structure:



wherein each occurrence of R^{1a} and R^{2a} is a biocompatible group and includes a carbon atom covalently attached to C^1; R^x includes a carbon atom covalently attached to C^2; n is an

integer; each occurrence of R^3, R^4, R^5 and R^6 is a biocompatible group and is independently hydrogen or an organic moiety; and for each occurrence of the bracketed structure n, at least one of R^{1a}, R^{2a}, R^3, R^4, R^5 and R^6 comprises a carbonyl group suitable for oxime formation.

13. **(Cancelled)**.

14. **(Previously Presented)** The conjugate of claim 12, wherein one or more occurrence of M is selected from the group consisting of proteins, antibodies, antibody fragments, peptides, antineoplastic drugs, hormones, cytokines, enzymes, enzyme substrates, receptor ligands, lipids, nucleotides, nucleosides, metal complexes, cations, anions, amines, heterocycles, heterocyclic amines, aromatic groups, aliphatic groups, intercalators, antibiotics, antigens, immunomodulators, and antiviral compounds.

15-18. **(Cancelled)**.

19. **(Original)** The conjugate of claim 1, wherein the conjugate is water-soluble.

20. **(Previously Presented)** The conjugate of claim 1, wherein the conjugate comprises a pharmaceutically useful modifier and a detectable label.

21-41. **(Cancelled)**.

41. **(Original)** A composition comprising the conjugate of claim 1 and a pharmaceutically suitable carrier or diluent.

42. **(Previously presented)** A composition comprising a conjugate of claim 1 associated with an effective amount of a therapeutic agent; wherein the therapeutic agent is incorporated into and released from said conjugate matrix by degradation of the conjugate matrix or diffusion of the agent out of the matrix over a period of time.

43. **(Previously Presented)** The composition of claim 42 wherein said conjugate is further associated with a diagnostic label.

44. **(Withdrawn)** A method of administering to a patient in need of treatment, comprising administering to the subject an effective amount of a suitable therapeutic agent; wherein said therapeutic agent is associated with and released from a conjugate of claim 1 by degradation of the conjugate matrix or diffusion of the agent out of the matrix over a period of time.

45. **(Withdrawn)** The method of claim 44 wherein said therapeutic agent is locally delivered by implantation of said conjugate matrix incorporating the therapeutic agent.

46. **(Withdrawn)** The method of claim 44 wherein said therapeutic agent is selected from the group consisting of: vitamins, anti-AIDS substances, anti-cancer substances, antibiotics, immunosuppressants, anti-viral substances, enzyme inhibitors, neurotoxins, opioids, hypnotics, anti-histamines, lubricants, tranquilizers, anti-convulsants, muscle relaxants and anti-Parkinson substances, anti-spasmodics and muscle contractants including channel blockers, miotics and anti-cholinergics, anti-glaucoma compounds, anti-parasite and/or anti-protozoal compounds, modulators of cell-extracellular matrix interactions including cell growth inhibitors and anti-adhesion molecules, vasodilating agents, inhibitors of DNA, RNA or protein synthesis, anti-hypertensives, analgesics, anti-pyretics, steroidal and non-steroidal anti-inflammatory agents, anti-angiogenic factors, anti-secretory factors, anticoagulants and/or antithrombotic agents, local anesthetics, ophthalmics, prostaglandins, anti-depressants, anti-psychotic substances, anti-emetics, imaging agents.

47. **(Withdrawn)** The method of claim 44 further comprising administering with the therapeutic agent additional pharmaceutically useful compounds selected from the group consisting of vitamins, anti-AIDS substances, anti-cancer substances, antibiotics, immunosuppressants, anti-viral substances, enzyme inhibitors, neurotoxins, opioids, hypnotics,

anti-histamines, lubricants, tranquilizers, anti-convulsants, muscle relaxants and anti-Parkinson substances, anti-spasmodics and muscle contractants including channel blockers, miotics and anti-cholinergics, anti-glaucoma compounds, anti-parasite and/or anti-protozoal compounds, modulators of cell-extracellular matrix interactions including cell growth inhibitors and anti-adhesion molecules, vasodilating agents, inhibitors of DNA, RNA or protein synthesis, anti-hypertensives, analgesics, anti-pyretics, steroidal and non-steroidal anti-inflammatory agents, anti-angiogenic factors, anti-secretory factors, anticoagulants and/or antithrombotic agents, local anesthetics, ophthalmics, prostaglandins, anti-depressants, anti-psychotic substances, anti-emetics, imaging agents, and combination thereof.

48. **(Withdrawn)** The method of claim 44 wherein said conjugate further comprises or is associated with a diagnostic label.

49. **(Withdrawn)** The method of claim 48 wherein said diagnostic label is selected from the group consisting of: radiopharmaceutical or radioactive isotopes for gamma scintigraphy and PET, contrast agent for Magnetic Resonance Imaging (MRI), contrast agent for computed tomography, contrast agent for X-ray imaging method, agent for ultrasound diagnostic method, agent for neutron activation, moiety which can reflect, scatter or affect X-rays, ultrasounds, radiowaves and microwaves and fluorophores.

50. **(Withdrawn)** The method of claim 48 wherein said conjugate is further monitored *in vivo*.

51. **(Withdrawn)** A method of administering a conjugate of claim 1 to an animal, comprising preparing an aqueous formulation of said conjugate and parenterally injecting said formulation in the animal.

52-53. **(Cancelled)**.

54. **(Withdrawn)** A method of administering a conjugate of claim 1 to an animal, comprising preparing an implant comprising said conjugate, and implanting said implant into the animal.

55. **(Withdrawn)** The method of claim 54, wherein said implant is a biodegradable gel matrix.

56. **(Withdrawn)** A method for treating of an animal in need thereof, comprising administering a conjugate as in claim 51 or 54, wherein said conjugate is associated with a pharmaceutically useful component.

57. **(Cancelled).**

58. **(Withdrawn)** The method of claim 51, wherein the pharmaceutically useful component is a gene vector.

59. **(Withdrawn)** A method for eliciting an immune response in an animal, comprising administering a conjugate as in claim 51 or 54, wherein said conjugate comprises an antigen modifier.

60-62. **(Cancelled)**.

63. **(Previously Presented)** The conjugate of claim 4, wherein one or more occurrences of L^{M1} independently comprises a 4-(N-maleimidomethyl)cyclohexane-1-carboxylate crosslinker.

64. **(Previously Presented)** The conjugate of claim 4, wherein one or more occurrences of L^{M1} independently comprises a m-maleimidobenzoyl crosslinker.

65. **(Previously Presented)** The conjugate of claim 4, wherein one or more occurrences of L^{M1} independently comprises a 4-(p-maleimidophenyl)butyrate crosslinker.

66. **(Previously Presented)** The conjugate of claim 1, wherein the molecular weight of the carrier is between about 1 and about 1000 kDa.

67. **(Previously Presented)** The conjugate of claim 11, wherein the molecular weight of the carrier is between about 1 and about 1000 kDa.

68. **(Previously Presented)** The conjugate of claim 12, wherein the molecular weight of the carrier is between about 1 and about 1000 kDa.

69. **(Previously Presented)** The conjugate of claim 1, wherein the carrier is hydrophilic.

70. **(Previously Presented)** The conjugate of claim 11, wherein the carrier is hydrophilic.

71. **(Previously Presented)** The conjugate of claim 12, wherein the carrier is hydrophilic.

Evidence appendix

Appellants provided the following evidence during prosecution of the instant application:

(1) Declaration by Mikhail I. Papisov, Ph.D., and Exhibits A, B, C,[3] D, and E. The Declaration and Exhibits A, B, C, D, and E were submitted along with a response to Office Action filed January 15, 2010, and were entered into the record in PAIR on the same date, designated "Rule 130, 131, or 132 Affidavits." Entrance into the record was confirmed by the Examiner reference to the Declaration on page 2 of the Office Action mailed April 30, 2010.

The **Declaration** is attached hereto on pages 29-36.

Exhibit A is attached hereto on pages 37-61.

Exhibit B is attached hereto on pages 62-73.

Exhibit C is attached hereto on pages 74-89.

Exhibit D is attached hereto on pages 90-99.

Exhibit E is attached hereto on pages 100-102.

[3] Exhibit C, Papisov, M.I., *ACS Symposium Series*. **786**, 301-314, 2001, was also submitted as "Appendix A" in the response to Office Action filed April 24, 2009, and was entered into the record in PAIR on the same date, designated "Appendix to the Specification." Entrance into the record was confirmed by the Examiner's reference to Appellant's April 24, 2009, submission on page 2 of the Office Action mailed July 15, 2009.

ATTORNEY DOCKET No.: 0492479-0041 (MGH 2170 US)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Papisov *et al.*	Examiner:	Yong Liang Chu
Serial No:	10/521,334	Group Art Unit:	1626
Filing Date	October 27, 2005	Confirmation No.:	1459
Title:	OXIME CONJUGATES AND METHODS FOR THEIR FORMATION AND USE		

Assistant Commissioner of Patents
Washington, DC 20231

Sir:

DECLARATION UNDER 37 C.F.R. § 1.132

I, Mikhail I. Papisov, hereby declare and state that:

1. I am an Associate Chemist and the Director/Principal Investigator (PI) of the Molecular Pharmacology and Pharmacological Imaging Laboratory at Massachusetts General Hospital, and Assistant Professor at Harvard Medical School. I have extensive research experience in the area of drug delivery, with particular expertise in the development of macromolecular pharmaceutical preparations, non-bioadhesive and selectively bioadhesive polymers and new bio-stealth materials and interfaces. A complete listing of my education and experience, including a list of publications I have authored, are summarized in my curriculum vitae, a true and accurate copy of which is provided with this declaration as **Exhibit A**.

2. I am a co-inventor on United States Patent Application Serial Number 10/521,334 filed on January 18, 2005 (the '334 application). I have reviewed and understood the Office Action from the US Patent and Trademark Office in the '106 application mailed July 15, 2009.

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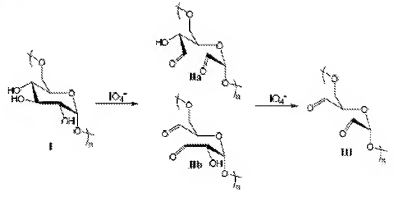
3. The purpose of the present Declaration is to describe the state of the art prior to the invention claimed in the present application. Specifically, the concepts of polyacetal stability and reactivity are discussed, and the differences between monomeric and polymeric functionalities is described.

4. To my knowledge, my laboratory was the first to study hydrophilic polyacetals, polyketals, and conjugates thereof. In fact, I am an inventor on three US Patents related to the polyacetal technology (see US. Pat. Nos. 5,811,510, 5,863,990, and 5,958,398). Such hydrophilic polyacetals are typically made via periodate oxidation of polymeric sugars to generate polymers comprising a polyacetal backbone decorated with carbonyl appendages, followed by a reduction that transforms some or all carbonyl groups into hydrophilic groups. As an example of the first step, illustrated in the specification on page 48 of the '334 application and reproduced below, a polyaldose of formula **I** (*e.g.*, dextran) undergoes periodate cleavage to generate alpha-hydroxy aldehydes **IIa** and **IIb**, which further oxidize to provide a polyacetal of formula **III**.



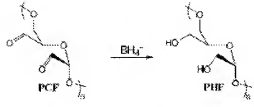
5. The structure drawn as formula **III**, also referred to as poly-[carbonylethylene carbonylformal] (PCF), is a simplified depiction of the actual structure of PCF. In reality, PCF exists as a composition of multiple structures in a pH-dependent equilibrium. For example, UV

studies reveal that at pH 4-5, most aldehyde groups of this type exist in a gem-diol form. At lower pH, the aldehyde absorption peak becomes apparent, while enol and enolate forms are present above pH 5.2. The enolate becomes especially prominent at pH > 7. The transitions between these four forms are not fast, and the actual tautomeric composition of the functional groups in the polymer of formula **III** may depend not only on the present pH, but on the pH to which the polymer had been exposed several hours earlier.

6. These characteristics of periodate-oxidized dextran have been known in the art since at least 1992 (see Drobchenko *et al.*, page 189, attached as **Exhibit B**, and Papisov 2001, page 5, attached as **Exhibit C**).

7. It is known that aldehyde groups (or their hydrated or tautomeric forms) can react with each other, for example, resulting in the formation of hemiacetals (see Ishak F and Painter TJ, attached as **Exhibit D**). In addition, it has been postulated that the enol form of PCF is prone to engage in intermolecular associations at pH 5-7 (Papisov 2001, page 5), which further complicates the ability to predict the structure and reactivity of the aldehyde groups. Thus, prior to the filing of the '334 application, the skilled artisan would have been cognizant that the structure of PCF is susceptible to change as the pH is adjusted, and that such changes would impact the reactivity of the pendant aldehyde groups.

8. To complete the formation of a hydrophilic polyacetal, PCF may be treated with a reducing agent such as sodium borohydride to reduce the pendant aldehyde groups to alcohols. Stoichiometric reduction of PCF generates poly-[hydroxymethylethylene hydroxymethylformal] (PHF):



9. The stability of the PHF main chain is pH-dependent. Size exclusion chromatography (SEC) studies have indicated that while incubation of PHF at neutral and high pH over several days does not change the SEC elution profile, incubation at pH < 7 showed significant fragmentation (see Papisov 2001, page 6, Figure 4).

10. There is ample literature describing an acid catalyzed (*i.e.*, pH-dependent) mechanism of hydrolysis of acetals as proton-dependent. However, my own studies suggest that the hydrolysis of polyacetals of the type being discussed is more complex and may also be general acid catalyzed. For example, in the presence of 50 mM sodium phosphate buffer at pH 3, the hydrolysis rate of the PHF main chain is double the rate of hydrolysis at pH 3 without phosphate buffer (see Papisov 2001, page 6). General acid catalysis is the most likely and the simplest explanation; however, the system is still incompletely studied and the mechanism of hydrolysis (which leads to depolymerization) may be even more complex. Any mechanism, however, would suggest that the polyacetal chain is sensitive not only to the pH, but also to the presence of external chemical entities.

11. As described above, aldehydes appended to a polyacetal main chain are in an equilibrium of several structures. When these aldehydes are in a gem diol or enol form, they become general acids. The enol form of these aldehydes is observed at pH > 5, and therefore even at relatively neutral pH, such polyaldehydes can be viewed as self-destabilizing. The same destabilization should be expected from any other general acids, such as protonated amines.

12. The present claims are directed toward biodegradable, biocompatible conjugates that contain one or more modifiers covalently attached to a polyacetal or polyketal carrier via oxime-containing linkages. One way of forming such conjugates is through the reaction of an aminooxy reagent with a polyacetal containing carbonyl groups suitable for oxime formation (such as PCF).

13. My own publication suggests that it might be possible to make conjugates of a polyacetal polymer (Papisov 2001, pages 7-8). However, even after considering this reference, one of ordinary skill would not be specifically motivated to produce conjugates as recited in the present claims. In particular, one of ordinary skill would not be motivated to select the one oxime-producing reagent mentioned in the 2001 reference, as a person having ordinary skill in the art would immediately recognize several likely perils of attempting to form an oxime bond with a polyacetal polymer. First, knowing that oxime formations are carried out under acidic conditions (oxime formation generally proceeds most quickly at pH ~ 4, as described in March, attached as **Exhibit E**), it would be apparent that the integrity of acetal groups of the polymer backbone would be an issue and their reactivity unpredictable. This is further complicated by the fact that one cannot predict *a priori* what pH will be optimal for the reaction due to the complex equilibrium of the aldehyde forms as described above. Second, if one chooses to use a modified PHF comprising aldehyde groups (as described in Papisov 2001 and shown in Scheme 4 on page 96 of the '334 application), the stability of PHF-like portions of the main chain would be subject to degradation below pH 7. It is important to note that these two points assume that the behavior of a modified PHF comprising aldehyde groups would retain the characteristics of its parts (*i.e.*, PCF and PHF). Since the art of synthetic chemistry is unpredictable, the skilled artisan would realize that the stability and reactivity of modified PHF comprising aldehyde groups under typical oxime-forming conditions is even less predictable than that of PCF or PHF alone. In fact, it is *completely unpredictable*.

14. Moreover, the very reagent used to form an oxime, a hydroxylamine, can itself destabilize the polymer main chain. Under oxime-forming conditions, the pKa of hydroxylamines is such that they are protonated species and thus general acids. Thus, there are two separate but equally problematic mechanisms for destruction of the polymer main chain under acidic conditions in the presence of a hydroxylamine used to form oximes.

15. The Office Action mailed July 15, 2009 cites two articles, Cervigni *et al.* and Rose *et al.*, as exemplifying reaction conditions for oxime formation that could be combined with the polyacetals of U.S. Patent No. 5,958,398 (the '398 patent). The conditions exemplified in these references include a pH of 3 or 4.6. Given the state of the art at the time of filing that I have described above, I can represent based on my experience that one of ordinary skill in the art would not have predicted that the presently claimed conjugates could have been made at any pH < 7, much less the pH levels described by these references.

16. Furthermore, the substrates used by Cervigni *et al.* and Rose *et al.* are monomeric aldehydes. The presently claimed conjugates are made using polymeric aldehydes. It is well known in the field of chemistry that functional groups may exhibit very different reactivity profiles in their monomeric and polymeric forms. For example, the pKa of monomeric (*e.g.*, acetic) acid is ~4.5-4.755, while the characteristic pKa of a polymeric carboxylic acid, depending on the formation of intramolecular hydrogen bonds and ionic strength of the solution, may be as high as is 6.5-7.5. It is widely known in polymer chemistry that in a polymer, intramolecular interactions change the local environment relative to that of a monomer, and thus the reactivity in monomeric form cannot necessarily be translated to polymeric form.

17. As any synthetic chemist knows, reactions performed on polymers must be held to a very high standard in terms of conversion and the avoidance of byproducts. This is because, unlike in small molecule reactions, byproducts form not as separate entities but as functional groups on the same polymer chains that contain the main products. Thus, polymers

contaminated with byproduct functionalities are typically impossible to purify. Therefore, when working with polymers the skilled artisan will choose to employ chemistry that is well-understood or at the very least compatible with the polymer.

18. In the context of the claimed conjugates, it is instructive to point out that a single hydrolytic break in the polymer main chain of each molecule renders a 50% reduction in the average molecular weight of the composition, two breaks render a 66% reduction, and so on. Thus, partial hydrolysis of the main chain results not in the formation of hydrolyzed byproducts that can be separated from the main product, but in polymers of a significantly lower molecular weight. For example, it is easy to see that a 1% hydrolysis of a polymer with polymerization degree n=100 leads not to 99% pure polymer (as it would be in the case of small molecules), but to a polymer with an average molecular weight reduced by a half. Since the molecular weight (size) is a critical parameter in certain biomedical applications, even "minor" hydrolysis can make the product biologically different, *e.g.*, inefficient or unsafe. Clearly, a skilled artisan concerned with both the purity and average molecular weight of the polymer conjugate composition would not consider subjecting a polyacetal of the '398 patent to the reaction conditions taught by Cervigni and Rose.

19. Due to all of the inherent stability and reactivity issues of polyacetals that were known at the time the '334 application was filed, I reiterate my position that a person of ordinary skill in the art would not have found the claimed conjugates to be obvious and achievable with a reasonable expectation of success.

20. To my knowledge, the first enabling disclosure of the presently claimed conjugates was in the '334 application.

21. Indeed, even my 2001 paper, which contains a statement that aldehyde groups "were conjugated with several model reagents via aldehyde condensation with amino-,

hydrazido-, aminooxy-, and other groups (see below)" points the reader to a later part of the document for a description of such conjugates (see Papisov 2001 pages 7-9). However, only conjugates of fMLFK-DTPA-PHF and polylysine graft copolymers are described as having been made. These aldehyde conjugation reactions were prepared via reductive condensation with amines, implying at a minimum that O-substituted hydroxylamines are not the first choice reagents for aldehyde condensation reactions, and that they can be used only under certain conditions that are not disclosed in the paper.

22. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

Mikhail I. Papisov, Ph.D.

Date: January 15, 2010

Exhibit A

CURRICULUM VITAE

I. GENERAL INFORMATION

Date prepared: January 15, 2010

Name: Mikhail I. Papisov

Office: Radiology BTH-506
Massachusetts General Hospital
Boston, MA 02114-2696
Phone: (617) 724-9655
Cell phone: (617) 967-4245
Fax: (617) 724-8315
Email: papisov@helix.mgh.harvard.edu

Residence: 60 Woodside Rd
Winchester, MA 01890
Phone: (781) 729-1384
Email: papisov@verizon.net

Place of Birth: Moscow, Russia

Education:

1976-1982	M.S. Chemistry	Moscow State University, Moscow, Russia
1982-1986	Ph.D. Biochemistry, Biology	Institute of experimental Cardiology, National Cardiology Research Center, Academy of Medical Sciences, Moscow, Russia

Postdoctoral Training:

1988-1989	Engineering Enzymology.	Laboratory of Engineering Enzymology Institute of Experimental Cardiology, National Cardiology Research Center, Academy of Medical Sciences, Moscow, Russia
1991-1993	Imaging	MGH NMR Center Massachusetts General Hospital and Harvard Medical School, Boston, MA.

Academic Appointments:

1989-1991	Research Scientist, National Cardiology Research Center, Academy of Medical Sciences, Moscow, Russia.
1993 -2005	Instructor in Radiology, Harvard Medical School, Boston, MA
2006 -	Assistant Professor of Radiology, Harvard Medical School, Boston, MA

Hospital or Affiliated Institution Appointments:

1989-1991	Head, Radiopharmaceutical Group	Dept. of Nuclear Medicine, Institute of Clinical Cardiology, National Cardiology Research Center, Academy of Medical Sciences of the USSR, Moscow, Russia
1993-2001	Assistant Chemist, Dept. of Radiology	Massachusetts General Hospital, Boston, MA
2001-2008	Fellow, Dept. of Research	Shriners Burns Hospital, Boston, MA
2001-	Associate Chemist, Dept. of Radiology	Massachusetts General Hospital, Boston, MA
2008-	Visiting Scientist	Shriners Burns Hospital, Boston, MA

Other Professional Positions:

1978	Senior Research Technologist	Division of Chemical Enzymology, Moscow State University, School of Chemistry, Moscow, Russia.
1989-1990	Head, Department of Applied Chemistry	Institute of Immunobiotechnology, (non-profit), Bioprocess Society, Academy of Sciences of the USSR, Moscow, Russia.
1990-1991	Associate Director, Board Member	Institute of Immunobiotechnology (private non-profit), Moscow, Russia.
2001-2002	Scientific Advisory Board	Puretech Ventures, Boston, MA
2001-	Scientific Advisory Board	Mersana Therapeutics, Cambridge, MA

Major Administrative Responsibilities

1990-1991	CEO, Board Member	Laboratory of Diagnostic Systems (non-profit) Academy of Medical Sciences, Moscow, Russia

Committee Service

2008-	Member, Research and Licensing Invention Liaison Program	Partners/MGH, Boston, MA

Professional Societies:

1994 -	Controlled Release Society, member
1999 -	American Chemical Society, member
2003-	Society of Nuclear Medicine, member
1999	Best Paper Award Committee, Controlled Release Society; member.

Grant Review Activities

2003-2004	NIH review board ZRG-1 SSS-K (10)
2004-2005	NIH review board ZRG-1 IDM-H (10)
2006-2007	NIH review board ZRG-1 IDM-G (10)
2009	NIH review board ZRG1 BST-M (58)

Editorial Activities

2001-	Cancer Research, reviewer
2001-	Biopharmaceuticals, reviewer
2001-	Materials Today/Nano Today, reviewer

Awards and Honors:

1993	Outstanding Pharmaceutical Paper, Controlled Release Society, Deerfield, IL (co-author)
1995	Outstanding Pharmaceutical Paper, Controlled Release Society, Deerfield, IL (first author)
2001	Scientific Founder, Mersana Therapeutics (form. Nanopharma Corp.), Cambridge, MA

II. RESEARCH, TEACHING AND CLINICAL CONTRIBUTIONS

A. Narrative report:

I focus my effort on the development of novel macromolecular drugs for human use, which makes **Investigation** (research) my major activity (70-80%over the last three years). The rest of my activities, 10-15% postdoctoral training, 5-10% administrative activities and 1-5% follow-up consulting on the developed technologies, are directly connected with research.

Investigation (research and technology development). My research centers on the development of "large molecule" drugs (therapeutic and diagnostic nanoparticles and macromolecules) and studies on the relationships between their structure, safety and efficacy.

My most significant contributions in this area were: (1) development of novel macromolecular materials (hydrophilic polyals) with a safety profile suitable for human use, and (2) based on these materials, development of novel macromolecular drugs, one of which has already entered clinical trials. The studies also included theoretical analysis and experimental investigation of macromolecule and nanoparticle behavior in vivo. These studies involved extensive use of imaging (single photon and PET), development of particle-specific mathematical pharmacokinetic models, and development of macromolecule/particle specific approaches in imaging and data analysis. Most recently, these approaches were utilized in an extensive imaging study of pharmacokinetics of five proteins in rats and monkeys (the proteins were prospective enzyme replacement therapeutics for children with genetic diseases). The study resulted in new information valuable for planning of the ongoing clinical trials (reported at two scientific conferences; full size papers are in preparation).

Research in the area of bioconjugate engineering and surface protection also resulted in several accessory technologies suitable for pharmacological applications. Many of these studies were carried out in collaboration with industrial research groups (Novartis, Amgen, Mersana, Intradigm, Berlex, ALZA/J&J), and resulted in new model preparations (macromolecular conjugates, non-viral gene vectors, siRNA carriers) that were subsequently used in drug development. All developed technologies were licensed to the pharmaceutical industry and generated significant research funds.

Teaching and Education. My activities in this area mostly consist of postdoctoral training of young scientists working under my supervision. The training (in the area defined as Molecular Pharmacology and Pharmacological Imaging) includes teaching of interdisciplinary methodologies related to large molecule engineering and characterization: synthesis, basics of structure-function relationships, cell culture and biological experiment, radionuclide and optical labeling, imaging. I also participate in the training of BS- and MS-level personnel as needed. As a member of Partners Invention Liaison Program, I also participate in consulting and mentoring in the area of intellectual property development and technology commercialization.

My other **activities** include follow-up consulting on chemistry, process engineering, quality control and biological evaluation of materials and technologies developed in my laboratory and licensed by MGH to the pharmaceutical industry. These activities peaked at the time of the founding of Nanopharma (presently, Mersana Therapeutics) – a start-up pharmaceutical company established to commercialize our technologies developed at MGH. To date, with completion of the information transfer and initial personnel training, these activities significantly subsided.

As the technologies developed over the last decade are being adopted by the industry, my research is shifting back to the investigation of large molecule behavior in vivo, mostly by PET imaging, and to the development of new technologies based on the new research results.

B. Funding Information (Research)

Past funded projects

1996-1997	PI	Inex Pharmaceuticals	Biomimetic stealth polymers in liposomal systems *Investigation of liposome stabilization and pharmacokinetics by hydrophilic polyacetals*
1996-1999	PI	The Whitaker Foundation	Surface Protection in Bioengineering *Modeling and experimental investigation of the behavior of molecular brushes on the surface of drug carriers and other interfaces*
1997-1999	Collab.	U.S. Army ("Idea" grant)	Peptide-targeted drug delivery to breast cancer PI: G-P. Dotto *Evaluation of random phage display libraries as a tool for identifying peptides enhancing drug delivery to breast cancer cells*
1998-1999	PI	Novartis/GTI	Fleximer technology in gene therapy (proof of principle) *Demonstration of the potential capabilities of Fleximer technology (hydrophilic polyacetals) to enhance stability of non-viral vectors in biological environments*
1999-2001	PI	Amgen	Biomimetic polymers for Invprotein modification *Investigation of protein modification (chemistry and pharmacokinetics) by hydrophilic polyacetals*
1999-2002	PI	Novartis/GTI	Fleximer technology in gene therapy *Development of non-viral stencely protected gene vectors based on hydrophilic polyacetals*
2000-2003	PI	NIH	Biodegradable hydrophilic polyacetals 1R21 RR14221-01A1 *Development of the technology of semi-synthetic and fully synthetic hydrophilic polyacetals and investigation of their properties*
2000-2003	Co-PI	DoE	Approaches to real-time imaging of mRNA transcripts DE-FG02-00ER63057 *Evaluation of approaches to develop a generic method for imaging gene expression via detecting mRNA transcripts in real time*

2002-2004	PI	Nanopharma Corp	Assay *Synthesis of four model drug conjugates with hydrophilic polyacetals for proof-of-principle studies at Nanopharma*
2002-2005	PI	NIH	Systemic Lymph Node-Specific Agents, 1 R41 AI052921-01 *Development of systemic lymph node-specific preparations for loading lymph node phagocytes infected with Category A pathogens with antibiotics*
2005-2006	PI	MGH ECOR	Safety of polymer-based nanoconstructs *Investigation of the underlying specific mechanisms that may result in toxicity of polymer-based nanoconstructs*
2006-2007	PI	Shire HGT	Radiolabeling and Pharmacokinetics of Replagal *Investigation of Replagal pharmacokinetics in rats after IV and SC administration by PET with Iodine-124. Replagal is a protein based enzyme replacement therapeutic for Fabri disease.*
2007-2008	PI	Shire HGT	Radiolabeling and Pharmacokinetics of ARSA *Investigation of human recombinant arylsulfase pharmacokinetics in rats after IV and IT administration by PET with Iodine-124. ARSA is a candidate enzyme replacement therapeutic for metachromatic leucodystrophy.*
2007-2008	PI	Shire HGT	Radiolabeling and Pharmacokinetics of Idursulfase *Investigation of Idursulfase pharmacokinetics in rats after IV and IT administration by PET with Iodine-124. Idursulfase is a candidate enzyme replacement therapeutic for Hunter syndrome.*
2007-2008	PI	Shire HGT	Radiolabeling and Pharmacokinetics of HNS *Investigation of human recombinant sulfamidase pharmacokinetics in rats after IV and IT administration by PET with Iodine-124. HNS is a candidate enzyme replacement therapeutic for Sanfilippo syndrome.*

Current funded projects

2007-2009	PI	Shire HGT	Replagal Pharmacokinetics in Non-human Primates *Investigation of Replagal pharmacokinetics in monkeys after IV and SC administration by PET with Iodine-124. Replagal is a protein based enzyme replacement therapeutic for Fabri disease.*
2008-2009	PI	Shire HGT	Pharmacokinetics of hGALC in Non-human Primates

Investigation of human recombinant galactosidase pharmacokinetics in monkeys after IV and IT administration by PET with Iodine-124 hGALC is a candidate enzyme replacement therapeutic for Globoid cell leukodystrophy (Krabbe disease).

C. Current unfunded projects

2005 -	PI	DNA as drug carrier	Development of macromolecular carriers for non-covalent DNA and binding drugs (intercalators, antisense oligonucleotides, siRNA)
2006-	PI	Vascular permeability in cancer and inflammation	Investigation of correlations between vascular permeability and drug efficacy
2006-	PI	Safety of polymer-based nanoconstructs	Investigation of key targets of nanoparticle toxicity
2008-	PI	Pharmacokinetics of large molecules in CSF and interstitial liquid	Investigation of large molecule translocations in cerebro-spinal fluid and interstitium

D. Report of Teaching and training:

1. Local (laboratory training):

Formally supervised postdoctoral trainees

1989-1990	Yury Arkhapchev, PhD	Chemistry; Natl. Cardiology Research Center, Moscow, Russia
2001	Andrey Talysin, M.D.	Molecular Pharmacology and Pharmacological Imaging; MGH, Boston, MA
2000-2003	Mao Yin, PhD.	Molecular Pharmacology and Pharmacological Imaging; MGH, Boston, MA
2000-2003	Mustafa Yatin, PhD	Molecular Pharmacology and Pharmacological Imaging; MGH, Boston, MA
2001-2005	Alex Yurkovetskiy, PhD	Molecular Pharmacology and Pharmacological Imaging; MGH, Boston, MA
2007-	Vasily Belov PhD	Molecular Pharmacology and Pharmacological Imaging; MGH, Boston, MA
2009-	Elena Belova, PhD	Molecular Pharmacology and Pharmacological Imaging; MGH, Boston, MA

Formally supervised PhD candidates

1989-1993	Larissa Popova, M.S.	Biochemistry; Natl. Cardiology Research Center, Moscow, Russia
1989-1991	Natalya Seregina, M.S.	Biology; Natl. Cardiology Research Center, Moscow, Russia

Formally supervised MS candidates

1989-1990	Irina Schipanova	Chemistry (MS project); Inst. of Fine Chemical Technology, Moscow, Russia
1991	Irina Majorova	Chemistry (MS project), Moscow State University, Moscow, Russia

Laboratory and Other Research Supervisory and Training Responsibilities

Clinical residents (amount of contact: 1-10 hours per week; experiment planning, protocol development, interpretation)

1991-1995	Residents involved in research at the MGH NMR Center	1-2 at a time, 1-10 hours per week
1991-	Laboratory training of BS-level personnel and pre-med students taking research year	1-3 at a time, amount of contact: 5-20 hours per week
2000-2001	Sungwoon Choi, PhD Postdoctoral	Molecular Pharmacology and Pharmacological Imaging; MGH, Boston, MA

Invited Lectures Local/Regional:

1996 University of Massachusetts. "Biodegradable biomedical polymers"

Invited Lectures National:

1995 "Magnetic nanoparticles: matrix synthesis and biomedical applications" 3M, TechForum, Minneapolis, MN

1996 "Long-circulating polyacetals" Genta Inc., San Diego, CA.

1996 "Biomimetic polymers" Amgen, Thousand Oaks, CA.

1997 "Approaches to novel diagnostic preparations" DuPont Merck Pharmaceutical Co.

1998 "Hydrophilic polyacetals" 7th Annual Meeting of the Bio/Environmentally Degradable Polymer Society, Cambridge, MA

2003 Hydrophilic Polyals: Biomimetic Biodegradable Stealth Materials. 226th Natl. Meeting of American Chemical Society, New York, NY, 2003

Invited Lectures International:

1989 'Magnetic Drug Transport' Magnetobiology conference, Sochi, Russia.

1990 'Magnetically guided drugs" Conference on electromagnetic field applications in medicine, Suhumi, Rep. of Georgia.

1996 'Biodegradable stealth polymers' Inex Pharmaceuticals, Vancouver, BC, Canada.

2005 Theoretical and Practical Aspects of Nano-Pharmacokinetics". Nanoparticles, international conference, org. by Center for Business Intelligence, Cleveland, OH.

E. Report of Clinical Activities

1988-1990 Development of infrastructure and personnel training for the newly established Radiopharmaceuticals Group, Dept. of Nuclear Medicine, Institute of Clinical Cardiology, National Cardiology Research Center, Academy of Medical Sciences of the USSR, Moscow, Russia

1999-2008 Methodological (laboratory) support of clinical research; assessment of the possibility of I-124 production at MGH and evaluation of ^{124}I as a label for PET. Division of Nuclear Medicine, Dept. of Radiology, MGH, Boston, MA.

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Research papers (peer reviewed):

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3. Papisov MI, Maksimenko AV, Torchilin VP. The optimization of reaction conditions during enzyme immobilization on soluble carboxyl-containing carriers. Enz. Microb. Technol. 1985; 7:11-6.

4. Rusetsky AN, Papisov MI, Ruuge EK, Torchilin VP. Substantiation of using magnet-directed localization of drugs for the treatment of thrombosis (Rus, Engl. abstract). Bull. USSR Cardiol. Res. Center 1985; 1:100-5.

5. Torchilin VP, Maksimenko AV, Tischenko EG, Papisov MI, Smirnov VN. Drug preparations of immobilized enzymes with enhanced affinity for the site of action (Rus). Antibiotiki I Biotehnologia 1986; 2:122-7.

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10. Papisov MI, Torchilin VP. Prediction of the biological effect of magnetically regulated drugs (Rus). Antibiotiki I Himioterapia 1988; N10:751-7

11. Papisov MI, Bogdanov AA, Schaffer BS, Nossiff N, Shen T, Weissleder R, Brady TJ. Colloidal magnetic resonance contrast agents: effect of particle surface on biodistribution. J of Magnetism & Magnetic Materials 1993; 122:383-6.

12. Weissleder R, Wang YM, Papisov MI, Bogdanov AA, Schaffer BS, Brady TJ, Wittenberg J. Polymeric contrast agents for MR imaging of adrenal glands. J Magn Res Imaging 1993; 3:93-97.

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2. Papisov MI. Modeling in vivo transfer of long-circulating polymers (two classes of long circulating polymers and factors affecting their transfer in vivo). Adv. Drug Delivery Rev., Special issue on long circulating drugs and drug carriers. 1995, 16:127-137.

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2. Papisov MI. Acyclic polyacetals from polysaccharides. ACS Symposium Series, vol. 786 (Biopolymers from polysaccharides and agroproteins), 2001, pp. 301-314.

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3. Torchilin VP, Ivanov NN, Klibanov AL, Papisov MI, Chebanov SM. On the mechanism of electron-dense liposome internalization by macrophages in vitro. In: Gregoriadis G, ed. Liposomes as drug carriers. Wiley, London, 1988; 63-74.

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8. Papisov MI, Weissleder R, Brady TJ. Diagnostic targeting of lymph nodes with polymeric imaging agents. In: Torchilin VP, ed. Targeted delivery of imaging agents. CRC Press, Boca Raton, 1995; 385-402.

9. Weissleder R, Bogdanov AA Jr., Papisov M. Drug Targeting in Magnetic Resonance Imaging. In: Torchilin VP, ed. Targeted delivery of imaging agents. CRC Press, Boca Raton, 1995; 133-148.

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57. M.I.Papisov, A.Hiller, S. Hillier, S.Sayed, A.J. Fischman. DNA as drug transport and release vehicle. 31th Int. Symp. on Controlled Release of Bioactive Materials, Honolulu, HI, 2004, Controlled Release Society, Deerfield, IL.

Abstracts, Poster Presentations and Exhibits Presented at Professional Meetings (last 3 years, not yet published as full length manuscripts)

1. M.I. Papisov, A.V. Yurkovetskiy, M. Yin, A. J. Fischman. Novel EPR-independent camptothecin conjugate with dual-phase drug release: a blood pool effect? 34th

Int. Symp. on Controlled Release of Bioactive Materials, Long Beach, CA, 2007. Controlled Release Society, Deerfield, IL.

2. M.I. Papisov, A.V. Yurkovetskiy, and A.J. Fischman. Pharmacokinetics of a novel camptothecin conjugate (PHF-CPT) with dual-phase drug release. Annual meeting of SNM, Washington, DC, 2007.

3. V. Belov, A. A. Bonab, A.J. Fischman, M. Papisov. Iodine-124 as a label for studying of slow pharmacokinetics. MGH ECOR Conference, February 2009.

4. M. Papisov, V. Belov, A. J. Fischman, A. A. Bonab, M. Wiles, H. Xie, M. Heartlein, P.Calias. PET Imaging of Enzyme pharmacokinetics in rats after IV and IT administration. Annual meeting of SNM. Toronto, CA. June 2009

5. M. Papisov, V. Belov, A. J. Fischman, A. A. Bonab, J. Titus, M Wiles, H. Xie, M. Heartlein, P.Calias. PET Imaging of α-Galactosidase A Pharmacokinetics in Rats and Monkeys. Annual meeting of SNM, Toronto, CA, June 2009

6. V. Belov, A. A. Bonab, A.J. Fischman, M. Heartlein, P. Calias, M. Papisov. Iodine-124 as a Label for pharmacological PET imaging. Annual meeting of SNM, Toronto. CA, June 2009

7. V. Belov, A. A. Bonab, A.J. Fischman, M. Papisov. Iodine-124 as a PET Imaging Label for Pharmacokinetic Studies. 36th Annual Meeting of the Controlled Release Society. Copenhagen, Denmark, July 2009

Patents (Report of Technological and Other Scientific Innovations)

Focus of activity

Development of "large molecule" (LM) therapeutic drugs suitable for human use is the focus of my research and innovations.

The LM drugs (macromolecules and particles of ca. 5-200 nm in diameter) have distinctive pharmacokinetics due to their limited renal clearance and selective extravasation through "small" and "large" vascular pores (the amount and function of the later varies in accordance with local pathological conditions).

Our ability to exploit the unique features of the pharmacokinetics of LM drugs and develop safe and effective therapeutic "nanoconstructs" is crucial for the development of therapeutics suitable for human use belonging to several classes, such as, gene vectors, siRNA, antisense oligonucleotides, large proteins, drug carriers and conjugates.

The below patents and applications (grouped in six IP packages) have been developed at MGH. All patents have been licensed to the pharmaceutical industry (Amgen, Novartis, Mersana Therapeutics) and the respective technologies evaluated preclinically and in clinical trials.

In summary, the results of this work are the following:

- A new company (Mersana Therapeutics, formerly Nanopharma) have been started in 2001, based entirely on the technologies developed by me or with my participation. The company licensed the below IP from MGH, and successfully raised two rounds of funding. Presently, the company has several LM drugs in development.
- One drug (a camptothecin-based macromolecular conjugate) is presently in Phase I trials and is expected to enter Phase II in 2009.
- Another drug, a macromolecular anti-angiogenic therapeutic, has been scaled up, investigated preclinically, and is expected to enter Phase I trials in 2009.
- A "pipeline" of drug candidates, also based on materials covered by the below patents, is under preclinical investigation at Mersana and is expected to enter human trials over the next several years.
- Nonviral gene vectors based on the technologies described below are being developed by Intradigm Inc. under MGH license.

Patents and applications are listed below, along with brief descriptions of the respective materials and technologies.

Patents

Group I. Systemic drug delivery to lymph nodes

This group of patents and applications cover drug carriers enabling systemic drug delivery to all lymph nodes (an spleen) through intravenous administration. Originally, the technology had been developed for delivery of T1 labels into lymph nodes for MR imaging. Presently, it appears that the technology can also be of value for delivery of antibacterial and antifungal agents to infected nodes, and, probably, for prevention of lymphatic metastasis. Accordingly, the technology was licensed to Winthrop-Sterling, transferred to Nycomed; currently the technology is being evaluated by Mersana Therapeutics under license from MGH.

1. Papisov MI, Brady TJ. System of drug delivery to the lymphatic tissues. US Patent 5,582,172, 12/10/1996.

2. Papisov MI, Brady TJ. System of drug delivery to the lymphatic tissues. Europe. Patent application 93918286.1. 7/21/1993
3. Papisov MI, Brady TJ. System of drug delivery to the lymphatic tissues. World Patent application WO9402068. February 3,1994.
4. Papisov MI, Brady TJ. System of drug delivery to the lymphatic tissues. Japan Patent JP7509467, October 19,1995
5. Papisov MI, Brady TJ. System of drug delivery to the lymphatic tissues. Australia Patent AU4778893, October 19,1995.

Group II Fleximer technology platform (Fleximer is a registered trademark of MGH)

This group of patents, applications and a trademark broadly covers two classes of structurally similar hydrophilic polymers, polyacetals and polyketals (polyals with intrachain oxygen). These materials were developed to provide biologically inert but biodegradable "stealth" materials for pharmacological applications.

Polyals are long-circulating and non-toxic; unlike polysaccharides (or other biomolecules) they don't induce anaphylactoid reactions, and unlike other long-circulating bioinert polymers (e.g., polyethylene glycol) they don't induce renal vacuolization.

Fleximer technology was evaluated by several pharmaceutical companies (chronologically, Inex, Amgen, Novartis, Mersana, and several others) with consistently positive conclusions. The first product based on Fleximer technology entered clinical trials in 2008. Several other new products (all are therapeutics for human use, mostly in oncology) are expected to be developed in the near future.

6. Papisov MI. Biodegradable polyacetal polymers and methods of their formation and use. US Patent 5,811,510, 09/22/1998.
7. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. US Patent 5,863,990, 01/26/99
8. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. US Patent 5,958,398, 09/28/99
9. Papisov MI. Biodegradable polyacetal polymers and methods of their formation and use. European Patent 0820473, 2001.
10. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. France, Patent 0820473, 6/27/2001
11. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. Germany, Patent 69613569.8-08, 6/27/2001
12. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. United Kingdom, Patent 0820473, 6/27/2001
13. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. Italy, Patent 0820473, 6/27/2001
14. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. Netherlands, Patent 0820473, 6/27/2001
15. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. Switzerland, Patent 0820473, 6/27/2001

16. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. Austria, Patent E292573, 6/27/2001
17. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. Belgium, Patent 0820473, 6/27/2001
18. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. Denmark, Patent 0820473, 6/27/2001
19. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. Finland, Patent 0820473, 6/27/2001
20. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. Greece, Patent 0820473, 6/27/2001
21. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. Ireland, Patent 0820473, 6/27/2001
22. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. Sweden, Patent 0820473, 6/27/2001
23. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. Japan, Patent 4187265, 9/19/2008
24. Papisov MI. Biodegradable polyketal polymers and methods of their formation and use. US patent application 20060069230; March 30, 2006.
25. Papisov MI. Biodegradable polyketal polymers and methods of their formation and use. European patent 1468036; October 15, 2008.
26. Papisov MI. Biodegradable polyketal polymers and methods of their formation and use. Australian patent application 2003209227; January 14, 2003.
27. Papisov MI. Biodegradable polyketal polymers and methods of their formation and use. World patent application 03/059988; January 14, 2003.
28. Papisov MI. Biodegradable polyacetal polymers and methods for their formation and use. Canada, Patent Application 2215997, 4/01/1996
29. Trademark "FLEXIMER" – registered by MGH to commercialize the above polyal technology.

Group III Conjugates of hydrophilic polyacetals

This group is dependent on Group II (uses Fleximer materials protected by Group II) and covers various Fleximer-protein conjugates. Patents 30-32 were filed upon the results of collaborative (MGH-Amgen) investigation of Fleximer conjugates with various proteins. Patents 33-34 were filed as a result of collaboration with Dr. Robson's group at Beth Israel Deaconess Medical Center.

30. Kinstler O, Ladd D, Papisov M Protein conjugates with a water-soluble biocompatible polymer. US Patent 7,160,924, January 9, 2007.
31. Kinstler O, Ladd D, Papisov M Protein conjugates with a water-soluble biocompatible polymer. World Patent application WO2004099774, January 29, 2004
32. Kinstler O, Ladd D, Papisov M. Protein conjugates with a water-soluble biocompatible polymer. Australia Patent application AU 2003256613, February 9, 2004
33. Elmaleh D., Robson S., Papisov M. Conjugates comprising a biodegradable polymer and uses thereof. US Patent Application 20050169968, August 4, 2005

34. Elmaleh D., Robson S., Papisov M. Conjugates comprising a biodegradable polymer and uses thereof. World Patent Application WO03/070823, August 28, 2003

35. Elmaleh D., Robson S., Papisov M. Conjugates comprising a biodegradable polymer and uses thereof. European Patent Application EP1585817, December 28, 2005, 2005

36. Papisov M.I. Protein conjugates with a water-soluble biocompatible, biodegradable polymer. US patent application 20080019940, January 24, 2008

Group IV Dual phase drug release system

This group of patents covers a new principle of drug delivery by polymer conjugates based on two-stage drug release: first as a highly hydrophobic prodrug from a hydrophilic conjugate, then as an active drug in cancer cells. The principle covered by this group is utilized in a Fleximer-conjugated camptothecin macromolecule that is currently under clinical investigation.

37. Papisov M., Yurkovetsky A. Dual phase drug release system. US Patent application 10/570,466, 3/2/06

38. Papisov M., Yurkovetskiy A. Polyacetal drug conjugates as release system. European Patent application 10/570,466, September 4, 2004

39. Papisov M., Yurkovetsky A. Dual phase drug release system. Japan Patent application 2007504253, March 01, 2007

40. Papisov M.I. Dual phase drug release system. US patent application 20070190018, August 16, 2007

41. Papisov M., Yurkovetskiy A. Dual phase drug release system. Australia, Patent application 2004270251, 2006/TBA

42. Papisov M., Yurkovetsky A. Dual phase drug release system. Europe, Patent application 04783400.7, 4/4/06

43. Papisov M., Yurkovetskiy A. Systeme de liberation de medicament en deux phase. Canada Patent application 2537993, June 3, 2006

Group V Nucleotide-based drug carriers

This group of patents covers a novel approach of drug molecule association with a macromolecule or nanoparticle through non-covalent association with a single or double strand of DNA or RNA. The approach is suitable for improving the pharmacokinetics of DNA and RNA binding drug substances, such as intercalators, antisense oligonucleotides, and siRNA. Currently under development at Mersana Therapeutics under license from MGH.

44. Papisov Mi. Drug-carrier complexes and methods of use thereof. US Patent 6,822,086, November 23, 2004

45. Papisov MI. Drug-carrier complexes and methods of use thereof. World Patent application WO0110468, February 2, 2001

46. Papisov Mi. Drug-carrier complexes and methods of use thereof. European Patent Application 00955415.5, 08/09/2000

47. Papisov MI. Drug-carrier complexes and methods of use thereof. Japan. Patent Application 2001514984, 08/09/2000

48. Papisov MI. Drug-carrier complexes and methods of use thereof. Japan Patent application JP2003506417, February 18, 2003

49. Papisov MI. Drug-carrier complexes and methods of use thereof. Australia Patent application AU6762600, March 5, 2005

50. Papisov MI. Drug-carrier complexes and methods of use thereof. US Patent application 20060019911, January 26, 2006

Group VI Oxime conjugates and linkers

This group covers an "accessory" conjugation technology suitable for selective drug conjugation with polymers of the Fleximer family (Group II) under mild conditions. The technology has been licensed to Mersana Therapeutics.

51. Papisov M., Yurkovetsky A. Oxime Conjugates, Methods of Preparation and uses Thereof", World Patent Application 2004/009982; January 29, 200

52. Papisov M., Yurkovetsky A. Oxime Conjugates, Methods of Preparation and uses Thereof", US Patent Application 20060058513; March 16, 2006

53. Papisov M., Yurkovetsky A. Oxime Conjugates, Methods of Preparation and uses Thereof", Europe, Patent Application 03765769.9, 2/16/05

54. Papisov M., Yurkovetsky A. Oxime Conjugates, Methods of Preparation and uses Thereof", Canada, Patent Application 2,492,803, 1/16/05

55. Papisov M., Yurkovetsky A. Oxime Conjugates, Methods of Preparation and uses Thereof", Japan, Patent Application 2006507232, March 2, 2006

56. Papisov M., Yurkovetsky A. Oxime Conjugates, Methods of Preparation and uses Thereof", Australia Patent Application 2003254023; February 9, 2004

Group VII.
Compounds for treatment of meningeal and neural diseases and methods of their use

57. M. Papisov. Compounds for treatment of meningeal and neural diseases and methods of their use. Provisional US Patent Application, filed June 12, 2009

58. M. Papisov and P. Calias. Equipment and method for drug delivery to the brain. IP Disclosure, submitted for filing, April 2009.

Exhibit B

Carbohydrate Research, 241 (1993) 189–199
Elsevier Science Publishers B.V., Amsterdam

An investigation of the structure of periodate-oxidised dextran

Svetlana N. Drobchenko [a], Ludmila S. Isaeva-Ivanova [a], Alexander R. Kleiner [a], Alexey V. Lomakin [a], Alexander R. Kolker [b] and Valentin A. Noskin [b]

[a] *St. Petersburg Nuclear Physics Institute, Russian Academy of Sciences, Gatchina SPb District 188350 (Russian Federation)*

[b] *Institute of New Technologies, Russian Academy of Sciences, St. Petersburg (Russian Federation)*

(Received February 12th, 1991; accepted June 23rd, 1992)

ABSTRACT

The aldo-enol transition in periodate-oxidised dextrans has been studied by UV absorption spectroscopy and electrophoretic light-scattering. Absorption peaks at 267, 240, and 290 nm are attributed to aldehyde, enol, and enolate ion, respectively. The electrophoretic mobility of periodate-oxidised dextran appears to be proportional to the absorption at 290 nm, and the pH dependence of the ratio of the peaks at 240 and 290 nm follows a standard titration curve. These facts are in accord with the formation of enol and enolate ions.

INTRODUCTION

The absence of UV and IR absorption for aldehyde groups in periodate-oxidised polysaccharides is usually attributed to the formation of hydrated hemiacetal and gem-diol groups[1]. However, for periodate-oxidised dextrans[2], the UV spectra depend on the pH of the solution, and only within a narrow range (4–5.2) is the absorption for aldehyde groups absent. Thus, at pH < 4 and > 5.2, there were peaks at 267 and 240 nm, respectively (Fig. 1). The peak at 267 nm is characteristic of aldehyde groups and that at 240 nm is assigned tentatively to an enol group. Likewise, the IR spectra of periodate-oxidised dextrans contain a typical aldehyde peak at 1740 cm^{-1} at pH < 4 and peaks at 1740 and 1620 cm^{-1} at pH > 5.2, with the latter assigned to the enol. These optical properties of periodate-oxidised dextrans do not correspond to any known structure. Arguments in favour of the enol form have been suggested[2] although, for 1,5-dicarbonyl compounds, such forms have not been reported.

Correspondence to: Dr. S.N. Drobchenko, St. Petersburg Nuclear Physics Institute, Russian Academy of Sciences, Gatchina SPb District 188350, Russian Federation.

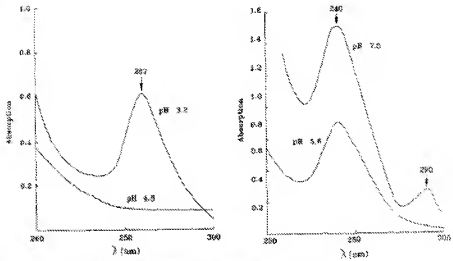


Fig. 1. UV spectra of periodate-oxidized dextrans as a function of pH.

We now report a more detailed study of this pH-dependent aldo → enol tautomerism. If an enol is formed at pH 5.2 then, at a higher pH, an enolate ion should be present. At pH > 7, a peak at 290 nm was observed and the shift (50 nm) from that at pH 5.2 is typical for an enol–enolate system[3]. In order to verify this interpretation, effects on the UV spectra and electrophoretic mobility[4] in the pH range 3–8 were studied.

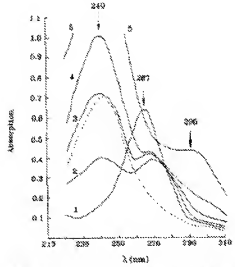


Fig. 2. UV spectra of periodate-oxidized dextran (60% oxidation, mol wt 60×10^3) as a function of time after a change in pH from 3 to 7. 1, zero; 2, after 2 h; 3, after 4 h; 4, after 6 h; 5, after 24 h; then after readjustment of the pH from 7 to 3 and 3-fold dilution (------).

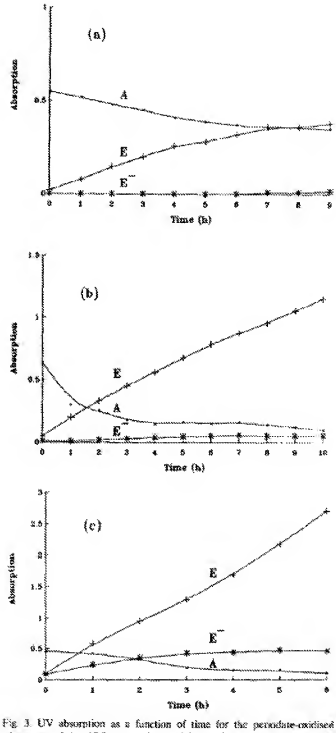


Fig. 3. UV absorption as a function of time for the periodate-oxidised dextran (see Fig. 2) after adjustment of the pH from 3 to (a) 6.5, (b) 7.0, (c) 7.7 at 267 (A), 240 (E), and 290 nm (E^{--}).

EXPERIMENTAL

Dextrans (Fluka) of molecular weights ($\times 10^3$) 20, 40, 60, 70, 110, and 500 were used. Each dextran was oxidised[2] with sodium periodate. Oxidation was carried out in glass-stoppered flasks protected from light. A solution of the dextran (2.4 g) in water (50 mL) was treated with 0.2 M sodium metaperiodate (50 mL, for 20% oxidation) for 24 h at room temperature at pH 4. After the excess of periodate had been destroyed with ethylene glycol, the solution was dialysed against running water for 24 h, then dialysed at pH 3 (acetate buffer) in order to remove products with molecular weights < 13000. Acetate ions were then removed by dialysis for a short time against water, and the solution was freeze dried. To a solution of each

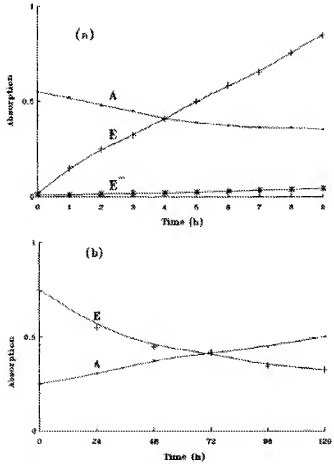


Fig. 4. UV absorption as a function of time for a periodate-oxidised dextran (40% oxidation, and at 60×10^{-3} I, after adjustement of the pH from 3 to (a) 7.5, and (b) back to 3 at 267 (A), 280 (E), and 290 nm (E').

periodate-oxidised dextran (6 mg) in water (1 mL) was added 0.01 M sodium phosphate buffer to give the desired pH in the range 6–9. UV spectra of the solutions were measured at intervals of 1 h or, in some experiments at high pH, at intervals of 10 min, with a Specord M-40 spectrophotometer. The aldehyde groups were determined by the iodine number[5], carboxyl groups by the method of Davidson[6], and enol by titration with bromine[7].

Electrophoretic mobilities were determined, with a Zeta Sizer II (Malvern Instruments) and an installation designed in St. Petersburg Nuclear Physics Institute, on solutions (10 mg/mL) in 30 mM sodium phosphate–citrate–borate buffer that contained 1% of NaCl, in order to obtain a conductivity of 2 mS/cm, and mM sodium azide for sterilisation. Each sample was centrifuged at 15000 rpm at 4°C for 1 h before measurements were made.

RESULTS

The UV absorption curves shown in Fig. 2 for the product derived from the dextran with a mol wt 60×10^3 are typical. At pH 3, there is only the aldehyde peak at 267 nm. As the pH is increased, the enol peak at 240 nm appears; finally, the peak at 290 nm becomes visible together with that at 240 nm. At high pH, the enol absorption shifts to greater wavelengths as enolate ions are formed[3]; hence, the peak at 290 nm can be assigned to an enolate ion. Since the above three peaks overlap, identification of the forms of the individual absorptions is necessary for quantitative evaluation. The curve for the aldehyde group is that at pH 3. However, at pH > 9, rapid irreversible destruction of the periodate-oxidised dextran occurred and the curve for the peak at 290 nm could not be determined. If, after several hours at pH 7.5, the pH of the solution was reduced to 3, the peak at 290 nm disappeared immediately, whereas that at 240 nm was restored slowly (dashed line in Fig. 2.) so that the absorption curve for the enol could be obtained.

TABLE I

UV absorption and analytical data for a solution (1 mg/mL) of 40% periodate-oxidised dextrans as a function of time at pH 7.5

Time after dissolution (h)	UV spectra				Analysis		
	A [a] (240 nm)	C=C–OH groups [b] (ε 2460) [c]	A (267 nm)	CHO groups [b] (ε 31) [c]	CHO groups [b]	C=C–OH groups [b]	COOH groups [b]
0			0.18	87	88	0	0.8
2	0.10	0.6	0.14	67	86	0.8	0.8
4	0.18	1.1	0.13	63	86	1.1	0.8
6	0.28	1.7	0.12	58	84	1.9	0.9
8	0.33	2.1	0.11	53	84	2.3	0.9
10	0.37	2.3	0.10	48	82	2.5	1.0

[a] Absorbance. [b] Per 100 residues. [c] Determined in a separate experiment

Fig. 3 shows a plot of the extinctions of the aldehyde, enol, and enolate groups versus time after increase of the pH from 3 to 6.5, 7.0, and 7.5. Fig. 4 shows the effect of reducing the pH from 7.5 to 3. The peak at 290 nm disappeared immediately; the peak at 240 nm reappeared first, followed by that at 267 nm. The extinction coefficients of the absorptions of the aldehyde and enol groups are quite different and a small proportion of enol contributes significantly to the absorption

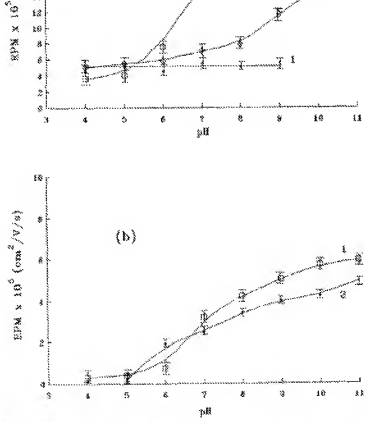


Fig. 5. Electrophoretic mobility (EPM) of periodate-oxidised dextrans as a function of pH: (a) 1, dextran (mol wt 500×10^3); 2, after 4% oxidation; 3, after 40% oxidation; (b) 1, 4% oxidised dextran of mol wt 20×10^3; 2, 4% oxidised dextran of mol wt 66×10^3.

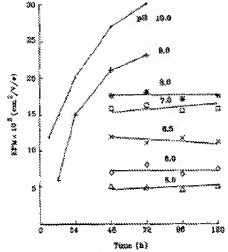


Fig. 6. Stability of the electrophoretic mobility (EPM) of periodate-oxidised dextran (mol wt 60×10^3, 40% oxidation) as a function of pH.

(see Table 1). The extinction coefficient for the aldehyde has a standard value (31), whereas that (2400) for the enol was 5 times less than normal.

In order to confirm the assignment of the peak at 290 nm to enolate ions, the charge on the periodate-oxidised dextrans was investigated by the electrophoretic light-scattering method. Fig. 5a shows that the electrophoretic mobilities of dextran and periodate-oxidised dextran at pH 4–5 are similar. This mobility depends on molecular weight. As the pH is increased, only the mobility of the periodate-

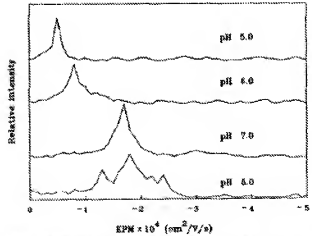


Fig. 7. Electrophoretic light-scattering spectra of periodate-oxidised dextrans as a function of the pH of the solution.

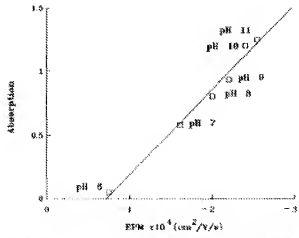


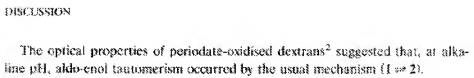
Fig. 8. UV absorption at 290 nm vs. electrophoretic mobility (EPM) for a periodate-oxidised dextran (mol wt 60×10^3, 8% oxidation) as a function of pH.

oxidised dextran increases in a manner that is roughly proportional to the extent of oxidation, and the effect of variation of molecular weight is negligible (Fig. 5b).

The electrophoretic mobility was practically independent of time at pH 5–8 (Fig. 6). At pH > 9, the mobility of extensively periodate-oxidised dextrans (> 20%) increased sharply and then became constant. For less-extensively oxidised dextrans (4–10%), there was no increase in mobility. That decomposition of the polysaccharides occurs at pH < 8 and results in the appearance of extra charges accords with the shape of scattered-light spectra. Fig. 7 shows that, at low pH, the sample was homogeneous with respect to charge, but that the distribution became broad at high pH. Fig. 8 shows a plot of the electrophoretic mobility against absorption at 290 nm as a function of pH.

DISCUSSION

The optical properties of periodate-oxidised dextrans[2] suggested that, at alkaline pH, aldo-enol tautomerism occurred by the usual mechanism (1 ⇌ 2).



1 2 3

At sufficiently high pH, the enol group loses a proton to give the enolate ion (3). However, a molecule of water could be lost[2] from the oxidised terminal residue (4 → 5), with formation of an unsaturated aldehyde that also absorbs at ~ 240 nm.

4 5

In spite of the relatively small proportion of end groups (2–4%), the aldehyde absorption could be significant due to the formation of conjugated bonds [the initial dextrans have 96–98% of (1 → 6) linkages]. The destruction processes also could yield products that absorb in this region. Thus, UV absorption at 240 nm does not prove that an enol is formed. In the absence of conjugation, the presence of two aldehyde groups in periodate-oxidised dextrans could promote enolisation by hydrogen bonding between the enol and aldehyde groups. Further, the two oxygen atoms attached to C-1 could promote the transfer of hydrogen (**1** → **2**) to give the enol.

The sequence **1** → **2** → **3** explains the major kinetic features of the system studied. As the pH is increased, the formation of enolate ions is promoted. The data in Figs. 3 and 4a illustrate this tendency. However, it may be that, after restoration of the pH to 3 from 7.5 (Fig. 4b), the enol does not disappear. The reverse reaction is slow and it is possible that, at low pH, a metastable state is observed both initially and after re-acidification from pH 7.5. The data in Table I show that only a small proportion of the aldehyde is converted into enol. However, the magnitude of the peak at 267 nm varies significantly, which reveals the existence of other reactions, most probably the formation of hydrated hemiacetal and/or gem-diol groups[1]. If the final pH is < 6, there is no increase in the peak at 240 nm, but that at 267 nm gradually disappears. The restoration of the aldehyde peak after re-acidification is connected with dehydration rather than with enol → aldehyde transformation. The accuracy of data plotted in Figs. 3 and 4a is insufficient for unambiguous determination of the rate constants. Nevertheless, it is possible to check quantitatively whether the assignment of the peaks at 240 and 290 nm to enol and enolate ion, respectively, accords with the observed pH dependence of their magnitudes. Since the rate of enol dissociation is rapid, the relation between the absorptions of the enol and enolate ion should be governed only by pH. If [E] and [E$^-$] are the concentrations of the enol and the enolate ion, respectively, then

$$[E^-]/[E] = K/[H^+],$$

where K is the dissociation constant. There is no reason for K to be markedly dependent on pH; hence, the ratio (R) of the absorptions at 290 and 240 nm should be nearly proportional to 10^{pH}, i.e.,

$$\log R = pH - pK + \log A,$$

where $pK = -\log K$ and A is the ratio of the extinction coefficients for enolate ion and enol. As shown in Fig. 9, where $\log R$ is plotted against pH, this proportionality is fulfilled. Since the parameter A is unknown, the pK of the enol

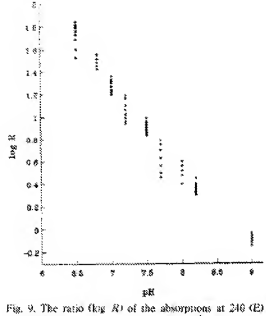


Fig. 9. The ratio (log R) of the absorptions at 240 (E) and 290 nm (E′) as a function of pH for periodate-oxidised dextran (mol wt 60×10^3, both 40 and 60% oxidation).

dissociation cannot be determined exactly. If it is assumed that the extinction coefficients for the absorptions of the enol and enolate ion are of the same order, i.e., A is ~ 1, then the pK will be ~ 7.5.

Electrophoretic light-scattering experiments support the above interpretation. Thus, at pH > 5, negatively charged groups appear on periodate-oxidised dextrans. These charges do not arise by oxidation of aldehyde to carboxyl groups, since[8] the pK for chelated 1,3-dicarbonyl compounds is 8–11 and that for carboxylic acids is 1–5. Thus, the electrophoretic behavior of periodate-oxidised dextrans (Fig. 5a, curve 3) is consistent with the formation of enolate ions. The distribution of electrophoretic mobility at pH > 8 is wide (Fig. 7). Since the mobility of a uniformly charged polymer is practically independent of its molecular weight[9], this effect could be caused by charged end groups created during decomposition of the polysaccharide. Therefore, the data have to be interpreted with caution. At pH < 8, no such problem exists and the electrophoretic mobility can be considered as proportional to the concentration of charged groups. Fig. 8 shows that the magnitude of the peak at 290 nm appears to be related linearly to the electrophoretic mobility. Thus, it is concluded that the absorption at 290 nm is due to enolate ions and that no other charged groups are present in significant proportion.

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Exhibit C

Acyclic polyacetals from polysaccharides

Biomimetic biomedical "stealth" polymers

M.I. Papisov

Department of Radiology, Massachusetts General Hospital and Harvard Medical School, Boston MA 02114-2696

Technologically adaptable hydrophilic polymers combining negligible in vivo reactivity with biodegradability would be instrumental in the development of specialized materials for advanced biomedical applications. Such highly biocompatible biodegradable polymers can be obtained via partial emulation of carbohydrate interface structures prevalent in biological systems. These structures are also present in polysaccharides and in some cases can be chemically "carved out" and isolated as acyclic hydrophilic polyacetals.

Introduction

Novel concepts in pharmacology and bioengineering impose new, more specific and more stringent requirements on biomedical polymers. Ideally, advanced macromolecular materials would combine negligible reactivity in vivo with low toxicity and biodegradability. Polymer structure should support an ample set of technologies for polymer derivatization; for example, conjugation with drugs, cell-specific ligands, or other desirable modifiers. Materials combining all the above features would be useful in the development of macromolecular drugs, drug delivery systems, implants and templates for tissue engineering.

On the chemistry level developing such materials translates into an intricate problem of developing macromolecules with minimized interactions in vivo, completely biodegradable main chains, and readily and selectively modifiable functional groups. The problem is further aggravated by the fact that both the main chain and the functional groups interact with extremely complex biological milieu, and all these interactions may be amplified via cooperative mechanisms.

Macromolecule interactions in vivo are mediated by several components of cells surfaces, extracellular matrix, and biological fluids. For example, both macromolecule internalization by cells and cell adhesion to polymer-coated surfaces

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can be mediated by several cell surface elements, many of which are functionally specialized (phagocytosis- and endocytosis-associated receptors, adhesion molecules, etc). Macromolecule recognition by cell receptors is often mediated by specialized recognition proteins of plasma, such as immunoglobulins (i), fibronectins (ii,iii,iv), proteins of complement system (v,vi,vii viii), soluble lectins (ix,x,xi,xii), vitronectin (xiii) etc. These proteins contain at least one receptor-recognizable site per molecule, and often more than one substrate-binding site. Although other proteins, e.g., albumin, also can bind polymers (via non-specific mechanisms), the distinctive features of recognition proteins relate to their ability to trigger remarkable biological responses. Some recognition proteins, such as C-reactive protein, are acute-phase proteins, i.e., their concentration in plasma increases as a result of inflammation or trauma. Others, such as β2-glycoprotein I (β2GPI), are "reverse" acute phase proteins, i.e., their concentration in blood during the acute phase decreases. Recognition proteins bind a variety of structures; we have reviewed their role in pharmacology of macromolecules and particulates in more detail elsewhere (xiv,xv).

Cooperative binding, often referred to as "non-specific interactions", is another major factor of macromolecule (and surface) reactivity in vivo. Cell interactions with polymers and recognition protein-polymer complexes also have an element of cooperativity (xvi,xvii). The very nature of cooperative interactions suggests that *any* large molecule can significantly interact with a complex substrate, for the simple reason that, because the binding energy is additive, the association constant of cooperative binding (K_a) would grow with the number of associations *exponentially* (xiv). In other words, any polymer of a sufficient length can be expected to interact with at least one of the various components of a biological system. Even if a molecule of certain size shows low interactions in cell cultures and in vivo, a larger molecule of the same type, or a supramolecular assembly, can have a much higher binding activity (xviii).

The essence of the above is that even if polymer molecules are assembled of domains that do not interact with cell receptors and recognition proteins, such molecules can be capable of cooperative interactions in vivo, i.e., completely inert polymers may not exist at all. However, several biomolecules and biological interfaces do appear to be functionally inert, except their specialized signaling domains. For example, plasma proteins are known to circulate for several weeks without uptake in the reticuloendothelial system (RES), whereas artificial constructs of a similar size have never been reported to have comparable blood half-lives.

Hypothetically, the mutual "inertness" of the natural biomolecules and surfaces may relate to their relatively uniform interface structures, where the potential binding sites are always saturated by naturally occurring counteragents present in abundance. Therefore, emulation of the common interface structures can result in a material that would not actively interact with actually existing binding sites because these sites would be pre-occupied by the natural "prototypes".

Poly- and oligosaccharides are the most abundant interface molecules expressed (as various glycoconjugates) on cell surfaces, plasma proteins, and proteins of the extracellular matrix. Therefore, interface carbohydrates appear to be the best candidates for structural emulation. The main objective of the emulation is to identify

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and exclude all structural components that can be recognized, even with low affinity, by any biomolecule, especially by cell receptors and recognition proteins.

All interface carbohydrates have common structural domains, which appear to be irrelevant to their biological function. An acetal group and two adjacent carbons are present in all carbohydrates, whereas the receptor specificity of each molecule depends on the structure and configuration of the glycol domains of the carbohydrate rings (Figure 1). We hypothesized that biologically inert ("stealth") polymers could be obtained using substructures that form the acetal side of the carbohydrate ring, i.e., the -O-C-O- group and the adjacent carbons. Although functional groups that are common in naturally occurring glycoconjugates (e.g., OH groups) can be used as substituents, the potentially biorecognizable combinations of these groups, such as rigid structures at C1-C2-C3-C4 (in pyranoses), must be completely excluded. Positioning of the acetal groups within the main chain would ensure polymer degradability via proton-catalyzed hydrolysis.

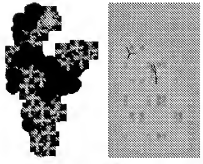

Figure 1 The structure of oligosaccharide interface fragment of glycolipid G_{M3} (space-filled and "stick" models of the same structure).

The signaling domains are shown in black; the biologically inert backbone in gray.

Materials of the suggested general structure (acyclic hydrophilic polyacetals) can be produced using a variety of methods. For example, cleavage of potentially biorecognizable fragments from all carbohydrate residues of a polysaccharide would result in acyclic structures similar to that of interface carbohydrates. We used exhaustive periodate oxidation to transform (1->6)-poly-α-D-glucose into acyclic poly[carbonylethylene carbonylformal] (PCF) with subsequent borohydride reduction resulting in poly[hydroxymethylethylene hydroxymethylformal] (PHF). Both polymers, PCF and PHF, were isolated and characterized in order to evaluate the viability of the concept.

Synthesis

Dextran B512, a product of *Leuconostoc Mesenteroides*, is a linear (1->6)-poly-α-D-glucose with ca. 5% (1->3; 6) branching; 95% of the branches are only one or two

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residues long (xix). Periodate oxidation of 1→6 connected polysaccharides has been previously studied (xx). In unsubstituted pyranosides the periodate reaction, which is highly specific to 1,2-glycols, starts from breaking either C2-C3 or C3-C4 bond with formation of dialdehyde IIa or IIb. In dextrans, the kinetically controlled IIa/IIb ratio is approximately 7.5:1 (xx). The subsequent, slower stage results in the cleavage of carbon C3, with formation of dialdehyde III (Figure 2).

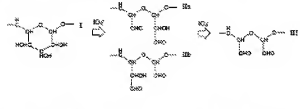

Figure 2. Exhaustive periodate oxidation of an unsubstituted pyranose ring

Thus, exhaustive oxidation of an entirely 1→6 connected polysaccharide is expected to occur without depolymerization, resulting in macromolecular poly-[carbonylethylene carbonylformal] (PCF). The aldehyde groups can be subsequently reduced with borohydride to obtain a hydroxymethyl-substituted polymer, poly-[hydroxymethylethylene hydroxymethylformal] (PHF, Figure 3).

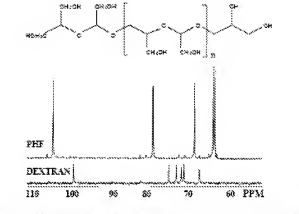

Figure 3. Poly[hydroxymethylethylene hydroxymethylformal] (PHF), structure and ^{13}C NMR spectrum, 295 K, 10% solution, 9.4 Tl Bruker system, 100.619 MHz by ^{13}C, proton decoupling, 45° flip angle, recycle delay 1.8 s (Dextran B512 spectrum is given as a reference).

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The ^{13}C NMR spectrum of the final product (Figure 3) confirms the expected structure and shows that, unlike some other dextrans, where complete oxidation is blocked (presumably, as a result of formation of intramolecular hemiacetals), Dextran B512 can be completely oxidized with no identifiable residual cyclic structures. The phenol-sulfate analysis (xxi) also showed only traces (<~0.1%) of the residual carbohydrate.

One of our practical objectives was to develop a technique for large scale polysaccharide processing without significant depolymerization. The major concerns related to (a) possible inclusions of non-1->6 linkages in the poly-4,1->6)-α-D-glucose main chain of Dextran B 512, that could be cleaved by periodate oxidation, and (b) relative instability of periodate-oxidized polysaccharides in alkaline media, which could result in depolymerization at the reduction stage (xxii). Preliminary tests showed that the commonly used versions of the periodate technique (developed for carbohydrate analysis and bioconjugate chemistry) afforded only small amounts of high molecular weight materials. Optimization of both the oxidation and reduction stages for minimal depolymerization resulted in consistently reproducible high yields of polymers with molecular weight distributions similar to the source dextrans (as determined by SEC HPLC) (xxiii,xxiv). Using flow dialysis as a prototype large scale technique for polymer purification and isolation, we obtained PHF with nearly theoretical yields for high molecular weight dextrans (MW>100 kDa). Low molecular weight polymers (MW~20-50 kDa) showed lower yields. The latter were attributed to inadequate polymer retention by low molecular weight cutoff filters, mainly at the final stage of PHF purification (PCF is reversibly associated in aqueous media, especially at 5<pH<7, which facilitates polymer retention by flow dialysis filters). Low molecular weight preparations of PHF were obtained with high yields via alternative procedures: (a) polymer purification by size exclusion chromatography, and (b) partial hydrolysis of 150-200 kDa polymers.

Properties

Both polymers, the intermediate PCF (Figure 2, III) and PHF (Figure 3), were obtained in >99% pure form (by SEC HPLC) as colorless solid compounds.

PCF was found to be stable in aqueous media below pH=6. Depending on the pH, PCF undergoes transitions that appear to be similar to the previously described for partially oxidized dextrans (xxv). At pH=4-5, most aldehyde groups seem to exist in a gem-diol form. At lower pH the aldehyde absorption peak (267 nm) becomes apparent, and above pH 5 both enol and enolate forms are present (240 and 290 nm). Formation of the enol form appeared to correlate with significant intermolecular association at pH=5-7. PCF was found to be soluble in water, dimethylsulfoxide (DMSO), dimethylformamide (DMFA), pyridine and water-alcohol mixtures, and insoluble in acetone, acetonitrile, dioxane, methanol, ethanol, glycerol, methylenechloride, toluene and triethylamine. Solubilization of dehydrated (lyophilized) preparations in water was slow, except at pH>7.

The reduced (polyalcohol) form, PHF, was found to be highly hygroscopic. Samples exposed to humid air were viscoelastic at ambient temperature. The apparent

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melting range of lyophilized PHF (MW=50-200 kDa) was within 100±20°C, depending on the molecular weight, and dramatically decreased after exposure to the ambient (humid) air. High molecular weight PHF is readily soluble in water, DMSO, DMFA and pyridine; slowly soluble in glacial acetic acid and ethyleneglycol, and insoluble in acetone, acetonitrile, dioxane, methanol, ethanol, glycerol, methylenechloride, toluene and triethylamine. Preparations with MW<5 kDa were soluble in methanol.

As expected, the stability of the PHF main chain was pH-dependent. While incubation at the neutral and high pH over several days did not change SEC elution profile, incubation at pH<7 showed significant fragmentation (Figure 4). In the presence of 50 mM sodium phosphate buffer, the hydrolysis rate at pH=3 was almost twice higher. Solubilization of crosslinked PHF gels in aqueous media showed an analogous pattern. At pH=7.5, both soluble and crosslinked PHF were resistant to a one hour incubation at 130°C.

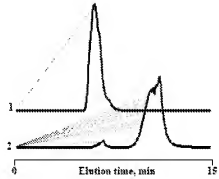

Figure 4. Size exclusion HPLC profile of 200 kDa PHF before (1) and after (2) 4 days incubation at pH=3, 37°C.

This pH dependence of main chain stability is valuable in several biomedical applications, where polymer-based products should be stable and functional in biological milieu (pH=7-7.5) but undergo depolymerization after internalization by cells. Degradation of the cell-internalized polymer is important to avoid adverse effects associated with long-term polymer deposition in cells, in the first place in the glomerular mesangium and reticuloendothelial system (xxvi,xxvii).

Acidic conditions (pH=5) are characteristic for the intracellular lysosomal compartment where polymers are transferred after internalization by cells. Therefore, cellular uptake of PHF-based preparations can be expected to result in non-enzymatic main chain hydrolysis at a moderate rate. This appears to be a significant advantage, as compared to several synthetic polymers, e.g., polyethyleneglycol, polyacrylates and vinyl polymers, which are hydrolysis-resistant. The final products of the PHF

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hydrolysis, glycerol and glycol aldehyde, have low toxicity, both are metabolized via major metabolic pathways. This may be one of the underlying reasons for the observed extremely low toxicity of PHF (see below).

Derivatives

Modification of either polymer did not present significant difficulties. Due to the availability of well-developed methods for alcohol and aldehyde group modification, the reaction conditions can be selected such as to ensure the integrity of the polyacetal main chain (e.g., at 4<pH<9 in aqueous media). Although neither polymer is soluble in most organic solvents, several desirable lipophilic derivatives, e.g., PHF conjugates with lipids, can be successfully synthesized in suitable solvent mixtures (e.g., pyridine-DMSO or pyridine-methanol).

To investigate the technological flexibility of PCF/PHF system and to characterize PHF-based preparations, several model linear and branched forms of derivatized PHF, model gels and bioconjugates were successfully synthesized and studied in vivo. The examples are given below.

PHF derivatization

Direct derivatization of PHF through primary alcohol groups. The alcohol groups of PHF can be acylated or alkylated in DMSO, DMFA or in water. Acylation with diethylenetriaminepentaacetic acid monocycloanhydride in DMSO was utilized to obtain PHF modified with diethylenetriaminepentaacetic acid (DTPA), a chelating group suitable for polymer labeling with metal ions such as ^{111}In (radioactive γ-emitter). Indium-111 labeled preparations were used in biokinetics and imaging studies. Alkylation with epybromohydrine in water was utilized to produce model epybromohydrine-crosslinked gels (that were used to investigate the resistance of PHF-based matrix to hydrolysis).

Derivatization through terminal 1,2-glycol group was used for producing terminus-activated PHF. The 1,2 glycol is formed at the former reducing end of the polysaccharide chain (whereas at the former non-reducing end a 1,3 glycol is present), see Figure 3. The 1,2-glycol is readily transformed into active aldehyde group via periodate oxidation. For example, a terminus-activated polymer with apparent molecular weight of 3.6±0.4 kDa per aldehyde group (I_2 titration) was produced and subsequently conjugated with lipids (in pyridine-methanol media) and proteins (in water) (xxviii).

Derivatization through non-terminal glycol groups. Non-terminal 1,2-glycol groups were introduced into PHF structure via modification of the polysaccharide oxidation technique. Oxidation of the original dextran was ca. 10% incomplete (all carbohydrate rings were open but 10% of the C3 were not eliminated), so the product of subsequent reduction (PHF) contained 1 glycol per 20 functional groups. The glycol groups were further oxidized with periodate resulting in PHF comprising active aldehyde groups along the main chain. The latter were conjugated with several model reagents via aldehyde condensation with amino-, hydrazide-, aminooxy- and other groups (see below).

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Partial fragmentation of the PHF backbone with simultaneous incorporation of new functional groups was used to produce PHF with activated terminal groups. Treatment with mercaptopropionic acid in DMFA (mercapolysan) resulted in fragments containing terminal carboxyls. The fragmented polymers were fractionated by precipitation (DMSO/chloroform or DMFA/acetone) and further subfractionated by HPLC. The terminal carboxylic groups were activated in DMSO with N-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide. The resultant polymer containing terminal N-oxysuccinimide ester group was precipitated and washed with chloroform and lyophilized. Terminal N-oxysuccinimido-PHF was used to produce soluble terminal graft copolymers (comb copolymers) with polyamines, e.g., with poly-L-lysine via direct reaction in water (xvii), and conjugates with lipids (dicteasroylphosphatidylethanolamine, DSPE) via condensation in DMSO/pyridine mixture. The DSPE-PHF conjugates were used for liposome stabilization (xviii).

PHF derivatives via modification of aldehyde groups of PCF

Modification of aldehyde groups of PCF (or PHF comprising aldehyde groups generated via glycol oxidation as described above) presents a set of synthetic approaches for producing a vast variety of PHF derivatives in mild conditions. For example, aldehyde groups can be conjugated in aqueous media with amines via formation of enamines with subsequent cyanoborohydride or borohydride reduction: this approach is widely used in protein immobilization on polymers (xxix,xxx).

Whenever conjugation through amines is not desirable, e.g., the reagent to be coupled with PHF has a biologically functional aminogroup, a variety of aldehyde group reactions with hydrazides, hydrazines, O-substituted hydroxylamines and 2-mercaptoamines (e.g., N-terminal cysteine) can be utilized. These reactions can be carried out in conditions where enamines are not formed (for example, in aqueous media at pH=6÷6).

Selectivity of aldehyde-mediated reactions opens the way to fast synthesis of complex functional conjugates, for example graft copolymers carrying multiple labels on the backbone (xxxi) and several cell-specific ligand groups (of one or more types) on the side chains. Aldehyde-mediated reactions can also be used for assembling complex PHF-based functional matrices e.g., for tissue engineering. Examples of PHF derivatization via aldehyde reactions are given below.

Partial derivatization of PCF was used to produce linear functionalized PHF derivatives and random-point PHF graft copolymers.

Linear PHF conjugate carrying fluorescein, DTPA and formyl-Met-Leu-Phe-Lys (f-MLFK, a chemotactic peptide) was synthesized via PHF condensation with cystamine (H_2N-C_2H_4-SS-C_2H_4-NH_2) and f-MLFK, with subsequent cystamine reduction and modification of the formed mercaptogroups with fluorescein isothiocyanate (fluorescent label) and DTPA (chelating group for ^{111}In). This preparation was used to

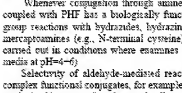

Figure 5. The structure of fMLFK-DTPA-PHF conjugate. The PHF backbone (ca. 1 kDa chain fragment shown) is modified by fMLFK (black) and DTPA (light grey) at random positions.

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a model cooperative vector for targeting formylpeptide receptors of white blood cells (xxxvii,xxxviii).

Random-point graft copolymers of PHF and DTPA-modified poly-L-lysine (backbone) were prepared, using previously developed technique (xxxi), via DTPA-Polylysine condensation with an excess of PCF, with subsequent reduction and separation of the unbound PHF. A dextran-polylysine graft copolymer was prepared analogously as a control for animal studies. The hydrodynamic size of both products, as determined by photon correlation light scattering, was 16±4 nm. Graft content was 20-25 molecules per backbone. Both copolymers were labeled with Indium-111 for animal studies.

In vivo studies

Because the central practical objective of this study was to develop a polymer with minimized interactions in vivo, we studied biokinetics of PHF and various PHF derivatives and attempted to identify the dose level at which toxic effects of PHF would become noticeable. Biokinetics provide valuable data on polymer interactions in vivo because, for particles and large macromolecules circulating in blood, blood half life is a mathematically exact measure of the overall polymer interactions with the biological milieu (xiv). Biologically inert ("stealth") polymers are expected to have insignificant accumulation in RES and other tissues. Low rates of tissue binding and uptake by cells result in a long blood half-life, except relatively small molecules (generally, MW<50 kDa) which can be cleared from blood via renal filtration.

Acute toxicity in mice. PHF of the highest molecular weight available at the time of the experiment (approximately 0.5-1 MDa) was used to minimize renal excretion that would mask the potential toxic effects. Although the injected dose reached 2 g/kg, all animals survived. After 30 days, all animals were alive, and their weights did not significantly differ from the control group (24±3 g vs. 25±2 g). None of the animals showed any noticeable symptoms of toxicity, including anaphylactoid reactions (e.g., paw edema) that develop in rodents in response to administration of many polymers, including dextran B 512. Absence of adverse reactions indicated that PHF interactions with immunocompetent cells and recognition proteins were biologically insignificant, which is in agreement with the underlying hypothesis. Administration of large doses of PHF-based preparations in rats and rabbits also did not cause any signs of toxicity nor anaphylactoid reactions.

Circulation of PHF was studied in normal anesthetized rats. Radiolabeled preparations were administered via tail vein. The initial biokinetics were studied by dynamic γ-scintigraphy (xxxii). Blood half-life of the low molecular weight [^{111}In]DTPA-PHF (50 kDa fraction) in rat was found to be 45 min (clearance via renal filtration). The polymer was cleared by 24 post injection, with very little accumulation in tissues (<0.05% dose/g in any tissue). The highest label accumulation (0.16% dose/g) was found in kidneys. The high molecular weight [^{111}In]DTPA-PHF (500 kDa fraction) demonstrated significantly longer circulation (blood half-life ca. 26 hr), with almost even distribution among tissues. Accumulation in RES was only

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twice as high as in other tissues, and thus was related, most likely, to a higher rate of spontaneous endocytosis in RES, rather than to PHF recognition by RES phagocytes.

Biokinetics of graft copolymers. Biokinetics of graft copolymers depend (at high graft densities) on the structure of the graft, whereas the effect of sterically hindered main chain is minimal. The graft copolymer model is sensitive to cooperative interactions because several graft chains can interact with a substrate (e.g., functional components of cell surface) simultaneously. For example, multiple chains of dextran B-512 in dextran/polylysine graft copolymers (and dextran-coated nanoparticles) are readily recognized by lymph nodes and spleen phagocytes, whereas single dextran molecules are not (xviii,xxxiii).

Biokinetics of graft copolymers were studied in normal outbred rats as described above. A series of graft copolymers of PHF with different graft densities showed the following results. Terminal (comb) copolymers with graft densities of two, seven, and ten PHF chains per backbone showed blood half-lives of 5.4±0.3, 7.3±1.2, and 9.8±1.5 hours, respectively. The long blood half-lives at higher graft densities, where copolymer molecule interactions are mediated essentially by the side chains, indicated low overall level of cooperative interactions of PHF in vivo.

In the subsequent comparative study, random-point graft copolymer of dextran showed blood half-life of ca. 1.5 hr. and a highly characteristic uptake in lymph nodes and spleen, with somewhat lower accumulation in liver and kidneys. Graft copolymer of PHF with analogous structure showed a much longer 25.3±2.5 hr. blood half-life, and a dramatically lower uptake in RES (Table 1).

Thus, the results of in vivo studies showed that neither linear nor highly branched PHF derivatives were efficiently recognized by RES, unlike the original Dextran B512. In studies with partially oxidized dextran (xxiii), loss of recognition correlated with elimination of the rigid stereospecific structures of the carbohydrate molecule.

Table 1. Biodistribution of Dextran and PHF graft copolymers in rat (% dose/g tissue), 24 hr. after intravenous administration (1 mg/kg body weight). From (xv).

Tissue	Graft	
	Dextran B-512	PHF
Blood	0.3	3.7
Lymph nodes, paraaortic	58.9	0.9
Lymph nodes, mesenteric	81.8	0.8
Spleen	19.9	1.3
Liver	9.0	2.1
Kidney	2.7	3.7
Muscle	0.1	0.4
Heart	0.3	0.9
Lung	0.2	1.2

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Biokinetics of PHF modified with chemotactic peptide was studied to evaluate PHF as a biodegradable "stealth" backbone polymer for targeted macromolecular drugs.

The model chemotactic peptide, f-MLFK, binds formylpeptide receptors of white blood cells. As a result, administration of labeled f-MLFK preparations results in label accumulation in the areas of white blood cell invasions, such as acute inflammations (xxxav). Peptide conjugation with macromolecules hypothetically can open the way to dramatic improvements in pharmacokinetics by means of (1) regulating the blood clearance via decreasing the rate of renal and, possibly, RES clearance and (2) increasing the agent-leukocyte association constant via cooperative binding effect of multiple peptide molecules exposed on the carrier. The cooperative character of agent-leukocyte interaction suggested an additional opportunity to explore a (3) hypothetical thermodynamic discriminatory effect that is expected to result in a more selective agent association with leukocytes and suppression of non-specific interactions with other tissues. The improvements in biokinetics, however, would be diminished if the backbone polymer interactions prevailed in the overall conjugate interactions in vivo.

Biokinetics of [^{111}In]DTPA-mercaptoethylamino-PHF-fMLFK, 15 and 70 kDa (Figure 5), was studied in a rabbits. Animals were normal or bearing focal bacterial inflammation induced by inoculation of E.Coli (clinical isolate) in thigh muscle. ^{111}In-labeled PHF-DTPA and monomeric DTPA-fMLFK were used as control preparations. Images were acquired over a 20 hr. period, followed by a biodistribution study.

The blood clearance rate of the 15 kDa preparation was fast; approximately 80% of activity was cleared from blood during the first 15 minutes through kidneys, the rest was cleared with a half-life of 45 min. The 70 kDa preparation showed half-life of 2 hr. with no initial fast phase. Both preparations significantly accumulated in the infection site. Scintigraphic images of the final biodistributions are shown in Figure 6.

Figure 6. Whole body scintigraphic images of rabbit (inflammation model).

Anterior view, 20 hr after administration of radiolabeled f-MLFK (left, control) and f-MLFK-PHF conjugate (right). K: kidneys; L: liver.

Note accumulation of both preparations in the inflammation (arrow), and significantly lower out-of-target accumulation of the f-MLFK-PHF conjugate, especially in kidneys.

The biodistribution data showed that immobilization of multiple f-MLFK molecules on PHF did not increase label accumulation in RES as compared to monomolecular f-

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MLFK, and decreased accumulation in kidneys by 80% (xxxvii). This study showed feasibility of PHF (from both technological and biological points of view) as a backbone polymer in targeted bioconjugates.

Discussion

The goal of this study was to determine whether a polymer emulating common acyclic structures of biological interface carbohydrates (hydrophilic polyacetal) would have a combination of properties close to an "idealized" biomedical material, such as: "inertness" in vivo, biodegradability of the main chain, low toxicity, and technological flexibility.

The model hydrophilic polyacetal, PHF, was produced via complete elimination of carbon 3 from carbohydrate residues of poly-(1→6)- α-D-glucose main chain of Dextran B-512. The blood clearance rates of PHF and PHF-protected macromolecules (graft copolymers) were close to that of similarly structured derivatives of polyethyleneglycol. (xxxv) which is currently the "gold standard" of biological inertness, and significantly longer than of analogously structured derivatives of dextran B-512 (xxxvi).

The potential advantages of hydrophilic polyacetals, as compared with polyethyleneglycol, are biodegradability and availability of readily modifiable groups along the main chain, which opens the way to producing various functional conjugates (xxxvii,xxxviii).

Advantages of polyacetals as compared to polysaccharides relate to both biological functionality and safety. For example, Dextran B512, (a.k.a. "pharmaceutical dextran"), which is known as one of the least biologically active polysaccharides (xxxix), is a product of a microorganism (*Leuconostoc Mesenteroides*). Dextran is known to produce anaphylactoid reactions that are mediated by immunoglobulins specific to isomalto-oligosaccharides (xl). The origin of the immunity is unknown; however, it has been shown recently that *Streptococcus Sanguinus*, an oral streptococcus prevalent in dental plaques (xli), produces isomalto-oligosaccharide containing hpotetchoic acid (xlii,xliii). The latter was shown to bind recognition proteins of plasma (xliv) and stimulate immunocompetent cells (xlv,xlvi). Therefore, *S Sanguinus* can potentially induce production of oligosaccharide-reactive antibodies, and the associated sensitivity to dextran-containing preparations, practically in any individual. Obviously, biomaterials lacking receptor-recognizable domains and antigenic determinants of wide-spread bacterial species would convey a much lower risk of anaphylactoid reactions.

Conclusion

The experimentally determined properties of the synthesized model acyclic hydrophilic polyacetal (PHF) were in a good agreement with the hypothesis that polymers obtained via partial oxidation of polysaccharides may have an excellent

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combination of useful features. Properties of PHF suggest the potential utility of polymers of this type in pharmacology and bioengineering, for example as structural or protective components in macromolecular drugs, drug delivery systems, and templates for tissue engineering. Development of carbohydrate-derived and fully synthetic hydrophilic polyacetals may become a promising direction in the development of new biomedical materials.

Acknowledgments

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Exhibit D

Carbohydrate Research, 64 (1978) 189–197

KINETIC EVIDENCE FOR HEMIACETAL FORMATION DURING THE OXIDATION OF DEXTRAN IN AQUEOUS PERIODATE

MONA FAHMY ISHAK* AND TERENCE J. PAINTER
Institute of Marine Biochemistry, N-7034 Trondheim-NTH (Norway)
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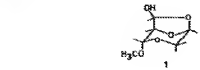
ABSTRACT

A kinetic analysis is described of the periodate oxidation of a dextran in which all the 93% of oxidisable D-glucose residues contained a 2,3,4-triol system. Measurements were made of the periodate consumed and the formic acid liberated by the dextran, the periodate consumed and the formaldehyde liberated by samples that had been partially oxidised and then reduced with sodium borohydride, and the glycerol and erythritol released from these samples by acid hydrolysis. Initially, the oxidisable D-glucose residues decayed according to second-order kinetics. After the first oxidative attack, ~40% of the singly oxidised residues very rapidly consumed a second mole of periodate, while the remainder consumed further periodate at about one-seventh of the rate of an intact D-glucose residue. Residues cleaved between positions 3 and 4 were generated 7.5 times faster than residues cleaved between positions 2 and 3, but the two kinds of singly oxidised residue subsequently decayed at similar rates. Towards the end of their reaction, the rate of decay of intact, oxidisable D-glucose residues declined in a way that was simply correlated with the proportion of doubly oxidised residues in the chains. A simple scheme is presented that explains these facts in terms of intra-residual hemiacetal formation by singly oxidised residues, and inter-residual hemiacetal formation between doubly oxidised residues and intact D-glucose residues adjacent to them in the chains.

INTRODUCTION

Yu and Bishop[1] observed that, when dextran was oxidised with periodic acid in methyl sulphoxide, it consumed only one mole of oxidant for every 1,6-linked D-glucose residue. After reduction of the product with sodium borohydride, acid hydrolysis yielded both glycerol and erythritol, and a similar oxidation of methyl β-L-arabinopyranoside afforded the hemiacetal **1**, identified as its crystalline acetate. These observations indicated that initial attack on the *trans-trans*-2,3,4-triol system in dextran was non-specific, and that a second attack was inhibited by spontaneous formation of the intra-residual hemiacetals **2** and **3**.

*Present address: The Chemical Department of Cairo, 12 Ramses Street, Cairo, Egypt.



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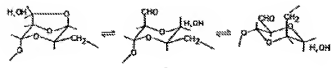

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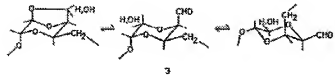

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We have investigated the possible formation of these and other hemiacetals in aqueous periodate, because of the importance of achieving complete oxidation in conventional, analytical oxidations of dextrans and other (1→6)-linked polysaccharides. One instance of a spuriously low oxidation-limit has already been reported for a dextran of very high molecular weight[2].

In principle, the required information could be obtained by n.m.r. spectroscopy of partially oxidised dextran in D_2O, but the number of different possible hemiacetal and hemialdal structures is formidably large, and it would be expected to vary with the degree of oxidation. The kinetic analysis now reported helps to simplify the problem, and provides a background for further work with n.m.r. and other methods.

EXPERIMENTAL

"Dextran 2000", having a weight-average molecular weight of $\sim 2 \times 10^6$, was supplied by Pharmacia Fine Chemicals AB, Uppsala, Sweden. It contained 0.42% of ash, which was corrected for, and was dried over phosphorus pentaoxide, *in vacuo* at 80°, before use. All reagents were of Merck analytical quality. Standard solutions were purchased in ampoules, and accepted as primary standards. The sodium metaperiodate was consistently ~99% pure by this criterion.

The analytical methods, and the method for preparing and reducing partially oxidised dextrans, were essentially as described for an earlier study of guaran[3], except that the volume of samples removed for titration of formic acid was increased to 25 ml. Analytical oxidations were carried out on 85-mg samples of dry dextran or reduced, partially oxidised dextran in 12.5mM sodium metaperiodate (200 ml) in the dark at 20.2°. The full course of the oxidation of dextran was studied by carrying

out a series of such oxidations in relays. Preparative oxidations were carried out under the same conditions as the analytical ones.

Samples (100 mg) of reduced, partially oxidised dextran were hydrolysed, in sealed tubes, in 0.25M sulphuric acid (2 ml) at 100° for 6 h. This was followed by neutralisation with barium carbonate, filtration, evaporation to dryness, and acetylation with acetic anhydride (2 ml) and dry pyridine (1 ml) at 80° for 1 h. In control experiments, artificial mixtures of erythritol and glycerol were treated similarly to convert chromatographic peak-area ratios into molar ratios. The inclusion of glycolaldehyde in these mixtures did not change the results.

The gas chromatograph was a Perkin–Elmer Model F11, coupled with a Model 165 recorder. Separation was effected on a stainless-steel column (2 m × 3 mm) filled with 1.5% Silicone XF-1150 and 1.5% poly(diethyleneglycol succinate) on acid-washed Chromosorb W (100–120 mesh). The flow-rate of nitrogen was 40 ml/min. A constant temperature of 110° was applied until glycerol triacetate was eluted, after which a linear gradient of 3.0°/min, up to 210°, was applied to elute erythritol tetra-acetate and α- and β-D-glucose penta-acetates. Samples were injected as solutions (1% w/v) in chloroform (1 μl). Peak areas were determined by weighing the peaks, excised from the paper.

RESULTS

The initial stages of the consumption of periodate (P_t) and the liberation of formic acid (F_t) by the dextran are shown in Fig. 1. Because the last part of the reaction was very slow, it is convenient to present the results for this part in tabular form, and this is done in Table I. The final consumption of periodate was 1.86 mol per D-glucose residue, and this yielded 0.93 mol of formic acid. All of the oxidisable residues therefore contained 2,3,4-triol systems.

Experiments were next carried out to determine whether the observed oxidation-limit was genuine, or spuriously low because of inter-residual hemiacetal formation[3,4]. Six samples of partially oxidised dextran were isolated after different periods of oxidation, reduced with sodium borohydride, and oxidised again. The results were corrected for the change in weight brought about by the release of formic acid in the first oxidation, and calculated on the basis of the intact D-glucose residues in the original dextran. They are shown, in part, in Fig. 1; in every case, a final oxidation-limit of 1.84 ±0.02 mol was indicated, in close agreement with the result obtained in the first oxidation.

The results for the second oxidations (Fig. 1) suggest that there was virtually instantaneous oxidation of D-glucose residues that had already suffered a single oxidative attack, and that this was followed by a much slower oxidation of the D-glucose residues that still remained intact in the samples. This view was confirmed by showing that the yield of formaldehyde in the second oxidation corresponded closely to the amount of rapidly consumed periodate (Table II). In addition, the

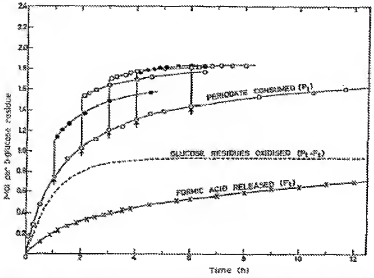

Fig. 1. Oxidation of dextran (5mM) in 12.5mM sodium metaperiodate at 20°. P_t and F_t are, respectively, the periodate consumed and the formic acid liberated at time t. At the points indicated by arrows, samples of partially oxidised dextran were isolated, reduced with borohydride, and oxidised again. The appended curves show periodate consumed by the samples.

initial slopes of the slow parts of the curves indicated a rate of oxidation similar to that of the original dextran.

Portions of the partially oxidised, borohydride-reduced samples were also hydrolysed with acid, and the products were acetylated and analysed for glycerol triacetate and erythritol tetra-acetate by g.l.c. The molar ratios (R) of the glycerol to the erythritol were too large for accurate measurement from the peak areas, but approximate values are given in Table II.

TABLE I

TERMINAL STAGES OF THE PERIODATE OXIDATION OF DEXTRAN[a]

t (h)	P_t	t (h)	F_t
14.67	1.67	14.00	0.733
16.67	1.69	16.00	0.766
18.67	1.71	26.00	0.844
19.67	1.72	36.00	0.930
21.67	1.73	38.30	0.930
23.67	1.76		
36.00	1.84		
38.30	1.85		
48.30	1.86		

[a]The experimental conditions and symbols are the same as for Fig. 1.

TABLE II

ANALYSIS[a] OF PARTIALLY OXIDISED DEXTRANS AFTER REDUCTION WITH BOROHYDRIDE

Time of first oxidation (h)	$(P_t - 2F_t)$ *in first oxidation*	IO_4^- *rapidly consumed*	*HCHO released*	*Molar ratio (R) Glyc/Ery*	R_{corr}[b]
1	0.306	0.338	0.356	13.4	7.4
2	0.425	0.435	0.427	15.2	8.0
3	0.448	0.460	0.420	17.1	8.4
4	0.425	0.415	0.410	16.4	7.3
5	0.392	0.390	0.380	17.8	7.3
6	0.371	0.370	0.370	18.6	7.1

[a]All quantities are calculated as mol per D-glucose residue in the original sample of unoxidised dextran. [b]Calculated from the formula $R_{corr} = [(P_t - 2F_t)R - F_t]/(P_t - F_t)$.

DISCUSSION

The numerical data provide a complete analysis of the composition of the reaction mixture at any time. Thus, P_t gives the concentration of periodate, F_t the mole fraction of doubly oxidised D-glucose residues, and $(P_t - 2F_t)$ the mole fraction of singly oxidised residues. The sum, $(P_t - F_t)$, which is the total fraction of D-glucose residues that have been oxidised at any time, is plotted in Fig. 1. An independent measure of the fraction of singly oxidised D-glucose residues is provided by the formaldehyde assays and the estimates of rapidly consumed periodate in the second oxidations, and the agreement with calculated values of $(P_t - 2F_t)$ is very good (Table II).

After correction for the glycerol originating from doubly oxidised D-glucose residues, the molar ratios of glycerol to erythritol (R_{corr} in Table II) indicate that residues cleaved between HO-3 and HO-4, and residues cleaved between HO-2 and HO-3, are generated in a ratio of ~7.5:1, respectively, and that they then undergo further oxidation at similar rates.

For the present purpose, the two most important quantities are P_t and $(P_t - F_t)$. By drawing tangents to the curve for P_t, and dividing their slopes by the concentration of residual periodate and the mole fraction of residual *vic*-diol groups $(2 - P_t)$, second-order rate-coefficients (k_p) for the consumption of periodate were calculated, and plotted against the degree of oxidation (Fig. 2, curve A). Similarly, slopes of tangents to the curve for $(P_t - F_t)$ were measured, and divided by the concentration of residual periodate and by $2[1 - (P_t - F_t)]$, to give second-order rate-coefficients (k_{ci}) describing the decay of intact, oxidisable D-glucose residues. These are also plotted in Fig. 2 (curve B).

Despite the considerable loss of accuracy that is involved in drawing tangents, the steady decline in k_{ci} with increasing degree of oxidation appeared to be significant, and an attempt was therefore made to correlate it with some other quantity that had been measured. It was found that the equation

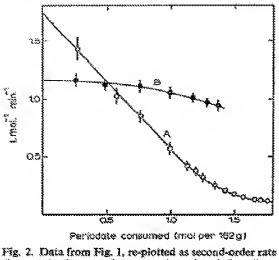

Fig. 2. Data from Fig. 1, re-plotted as second-order rate coefficients against the degree of oxidation. Curve A is the rate of consumption of periodate (k_G), and curve B the rate of decay of intact D-glucose residues (k_H).

$$k_G = 1.17(1 - 0.4\,F_1)\ \text{l.mol}^{-1}.\text{min}^{-1}$$

accounted reasonably well for the changes in k_G.

The dramatic decrease in k_G, during the initial period when k_G is changing very little (Fig. 2), clearly implies formation of the intra-residual hemiacetals **2** and **3**, provided one can assume that the singly oxidised residues, in their acyclic forms, are oxidised very rapidly, as expected from their behaviour after reduction (Fig. 1). Two facts must be noted: (*i*) the initial rate of consumption of periodate is ~40% higher than the initial rate of decay of intact D-glucose residues (Fig. 2); and (*ii*) the curve (F_1) for the liberation of formic acid (Fig. 1) does not show an induction period.

From a consideration of the theory of consecutive reactions[5], it is possible to appreciate that this situation can only come about when the rate of a second step is vastly greater than that of the first. We accordingly suggest that it is only possible to explain all of the facts in terms of the general reaction scheme shown in Fig. 3. The essential feature of this scheme is that, after the first oxidative attack, a singly oxidised residue subsequently reacts by one of two competing pathways, both of which are very fast.

(*a*) Ring-closure to give an unoxidisable, intra-residual hemiacetal. The possibility that a periodate ion may be involved in an unreactive complex with this hemiacetal should perhaps not be overlooked. The hemiacetal eventually reaches a state of equilibrium with the rapidly oxidisable, acyclic form, and attainment of the correct, Malapradian oxidation-limit is only possible because a minute amount of this form is always present at equilibrium.

(*b*) Consumption of a second mole of periodate before the equilibrium condition is reached.

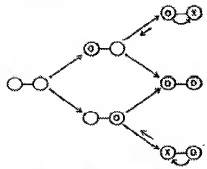

Fig. 3. Schematic representation of the periodate oxidation of an alicyclic *vic*-triol. Pairs of circles represent the two adjacent, oxidisable sites; "O" signifies that a site has been oxidised; "X" represents a site that is protected from oxidation by hemiacetal formation; and curved arrows represent hemiacetal rings.

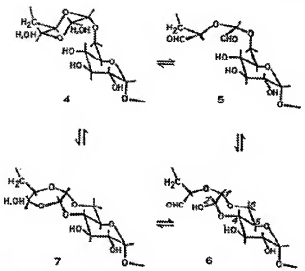

Fig. 4. Suggested explanation for the correlation between the rate of decay of intact D-glucose residues (k_G) and the proportion of doubly oxidised D-glucose residues (F_2) in the chains. It is postulated that the two kinds of residue have to be adjacent, as in **5**, and that oxidation between HO-3 and HO-4 of the intact residue is inhibited by formation of **6** and **7**. The dioxepane ring in **6** and **7** is shown in the $^{5,4}TC_{0,5}$ conformation, with substituents at C-2 isoclinal to the reference plane.

Our interpretation (Fig. 4) of the decline in k_G as the fraction of doubly oxidised residues increases is necessarily more tentative, since it does not rest upon the firm identification of a model hemiacetal corresponding to **1**. We have, however, reported other evidence[4] to show that seven-membered hemiacetal rings exist to a significant extent in aqueous solution when there is no possibility for the competitive

formation of a six-membered hemiacetal by the same aldehyde group*. On the other hand, when there is a possibility for forming a six-membered hemialdal, such as 4, in apparent competition with a seven-membered hemiacetal, the latter is still detectable[4]. This may be because hemialdals are fundamentally unstable in water (*cf.* Ref. 8), but it should be noted that both rings could be freely incorporated into a composite structure such as 7.

Fig. 4 accordingly shows formation of a seven-membered, inter-residual hemiacetal between the aldehyde group derived from C-2 of a doubly oxidised D-glucose residue, and HO-4 of an intact D-glucose residue adjacent to it in the chain. This would block the more reactive of the latter's two oxidisable sites. The proposed structure is conformationally plausible, with the 1,4-dioxepane ring as a twist-chair, and bulky substituents either equatorial or isoclinal. Formation of a similar hemiacetal between the aldehyde group derived from C-4 of a doubly oxidised residue and HO-2 of an intact one is less likely, because the two rings would be *cis*-fused, and encounter a severe "H-inside" interaction.

None of the hemiacetals considered here is sufficiently stable to give rise to an absolutely anomalous periodate-oxidation limit. The different results reported by Yu and Bishop[1] for oxidation in methyl sulphoxide must reflect the inability of this aprotic solvent to stabilise the oxidisable, acyclic forms of the singly oxidised residues by solvation of the free aldehydic groups (*cf.* Ref. 8). The same effect must also enhance the rate of cyclisation, relative to the rate of oxidation of the acyclic forms, in order to give the observed oxidation-limit of 1.0 mol of periodate consumed[1].

The present results do not help to explain the spuriously low limit reported by Leonard and Richards[2] for oxidation, in water, of a dextran of very high molecular weight. These authors associated the phenomenon with an observed tendency for the dextran to exist in solution as aggregates. Such a tendency might not only modify the reactivity of the D-glucose residues, but would also introduce the possibility of intermolecular hemiacetal formation.

ACKNOWLEDGMENT

The authors are much indebted to Bjørn Larsen for help and advice in performing the g.l.c. analyses.

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3 M. F. ISHAK AND T. J. PAINTER, *Acta Chem. Scand.*, 27 (1973) 1268–1276.
4 M. F. ISHAK AND T. J. PAINTER, *Acta Chem. Scand.*, 25 (1971) 3875–3877.
5 A. A. FROST AND R. G. PEARSON, *Kinetics and Mechanism*, Wiley, New York and London, 2nd edition, 1961, pp. 166–184.

*This is supported by the recent work of Grindley *et al.*[6], on substituted aldohexoses. Anot has also reported on septanose formation in aqueous solution[7].

6 T. B. GRINDLEY, V. GULASEKHARAM, AND D. B. TULSHIAN, *Abstr. Papers*, 2nd Joint CIC/ACS Conference, Montreal, Canada, May 29–June 2, 1977, CARB 12.
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Exhibit E

MARCH'S ADVANCED ORGANIC CHEMISTRY

REACTIONS, MECHANISMS, AND STRUCTURE

FIFTH EDITION

Michael B. Smith
Professor of Chemistry, University of Connecticut

Jerry March
Professor of Chemistry, Adelphi University

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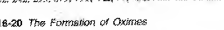
OS II, 395; **III**, 96, 351; **IV**, 351, 377, 536, 884; **V**, 27, 258, 747, 929; **VI**, 10, 12, 62, 242, 293, 679, 791; **VII**, 77, 438. Also see OS **III**, 708; **VI**, 161; **VIII**, 597.

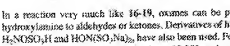
16-20 *The Formation of Oximes*

HYDROXYIMINO-DE-OXO-BISUBSTITUTION



In a reaction very much like **16-19**, oximes can be prepared by the addition of hydroxylamine to aldehydes or ketones. Derivatives of hydroxylamine, for example, H_2NOSO_3H and $HON(SO_3Na)_2$, have also been used. For hindered ketones, such as hexamethylacetone, high pressures (e.g., 10,000 atm) may be necessary.[714]

It has been shown[715] that the rate of formation of oximes is at a maximum at a pH that depends on the substrate but is usually ~4, and that the rate decreases as the pH is either raised or lowered from this point. We have previously seen (p. 425) that bell-shaped curves like this are often caused by changes in the rate-determining step. In this case, at low pH values step 2 is rapid (because it is acid catalyzed), and step 1



is slow (and rate determining), because under these acidic conditions most of the NH_2OH molecules have been converted to the conjugate NH_3OH^+ ions, which cannot attack the substrate. As the pH is slowly increased, the fraction of free NH_2OH molecules increases and consequently so does the reaction rate, until the maximum rate is reached at about pH = 4. As the rising pH has been causing an increase in the rate of step 1, it has also been causing a *decrease* in the rate of the acid-catalyzed step 2, although this latter process has not affected the overall rate since step 2 was still faster than step 1. However, when the pH goes above ~4, step 2 becomes rate determining, and although the rate of step 1 is still increasing (as it will until essentially all the NH_2OH is unprotonated), it is now step 2 that determines the rate, and this step is slowed by the decrease in acid concentration. Thus the overall rate decreases as the pH rises beyond ~4. It is likely that similar considerations apply to the reaction of aldehydes and ketones with amines, hydrazines, and other nitrogen nucleophiles.[716] There is evidence that when the nucleophile is 2-methylthiosemicarbazide, there is a second change in the rate-determining step: above pH about 10 *hydroxide* catalysis of step 2 has increased the rate of this step to the point where step 1 is again rate determining.[717] Still a third change in the rate-determining step has been found at about pH = 1, showing that at least in some cases step 1 actually consists of two steps: formation of a zwitterion (e.g., $HOH_2\overset{+}{N}-\overset{|}{C}-O^-$) in the case shown above, and conversion of this to **16**.[718] The intermediate **16** has been detected by NMR in the reaction between NH_2OH and acetaldehyde.[719]

In another type of process, oximes can be obtained by passing a mixture of ketone vapor, NH_3, and O_2 over a silica-gel catalyst.[720] Ketones can also be converted to oximes by treatment with other oximes, in a transoximation reaction.[721]

OS **I**, 318, 327; **II**, 70, 204, 313, 622; **III**, 690; **IV**, 229; **V**, 139, 1031; **VII**, 149. See also OS **VI**, 670.

16-21 *The Conversion of Aldehydes to Nitriles*

NITRILO-DE-HYDRO,OXO-BISUBSTITUTION

Aldehydes can be converted to nitriles in one step by treatment with hydroxylamine hydrochloride and either formic acid,[722] SeO_2,[723] or pyridine–toluene.[724] The reaction is a combination of **16-20** and **17-30**. Direct nitrile formation has also been accomplished with certain derivatives of NH_2OH, notably, NH_2OSO_3OH.[725] Another method involves treatment with hydrazoic acid, though the Schmidt reaction (**18-16**) may compete.[726] Aromatic aldehydes have been converted to nitriles in good yield with NH_2OH/HCOOH on silica gel[727] or NH_2OH on Mexican bentonite[728] with microwave irradiation, dimethylhydrazine followed by dimethyl sulfoxide,[729] with trimethylsilyl azide,[730] and with hydroxylamine hydrochloride, $MgSO_4$, and $TsOH$.[731]

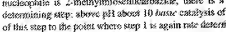
On treatment with two equivalents of dimethylaluminum amide (Me_2AlNH_2), carboxylic esters can be converted to nitriles: $RCOOR' \rightarrow RCN$.[732] This is very likely a combination of **10-58** and **17-32**. See also **19-5**.

OS **V**, 656.

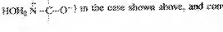
F. *Halogen Nucleophiles*

16-22 *The Formation of gem-Dihalides from Aldehydes and Ketones*

DIHALO-DE-OXO-BISUBSTITUTION



Aliphatic aldehydes and ketones can be converted to *gem*-dichlorides[733] by treatment with PCl_5. The reaction fails for perhalo ketones.[734] If the aldehyde or ketone has an α hydrogen, elimination of HCl may follow and a vinylic chloride is a frequent side product.[735]



Related proceedings appendix

None.

Conclusion

Appellants conclude with the belief that claims 1-6, 11, 12, 14, 19, 20, 41-43, and 63-71 are patentable. Allowance of the pending claims is earnestly requested.

Please charge any additional fees that may be associated with this matter, or credit any overpayments, to our Deposit Account No.: 03-1721.

Respectfully submitted,

/Brenda Herschbach Jarrell/

Dated: November 8, 2010

Brenda Herschbach Jarrell, Ph.D., J.D.
Reg. No.: 39,223

Choate, Hall & Stewart LLP
Patent Group
Two International Place
Boston, MA 02110
bjarrell@choate.com
Tel: 617-248-5175
Fax: 617-248-5002